Retinoid X Receptor Agonists Increase Bcl2a1 Expression and Decrease Apoptosis of Naive T Lymphocytes[1]

Reuven Rasooly,* Gertrud U. Schuster,* Jeffrey P. Gregg,† Jia-Hao Xiao,‡ Roshantha A. S. Chandraratna,‡ and Charles B. Stephensen[2]*

Vitamin A affects many aspects of T lymphocyte development and function. The vitamin A metabolites all-*trans*- and 9-*cis*-retinoic acid regulate gene expression by binding to the retinoic acid receptor (RAR), while 9-*cis*-retinoic acid also binds to the retinoid X receptor (RXR). Naive DO11.10 T lymphocytes expressed mRNA and protein for RAR-α, RXR-α, and RXR-β. DNA microarray analysis was used to identify RXR-responsive genes in naive DO11.10 T lymphocytes treated with the RXR agonist AGN194204. A total of 128 genes was differentially expressed, including 16 (15%) involved in cell growth or apoptosis. Among these was Bcl2a1, an antiapoptotic Bcl2 family member. Quantitative real-time PCR analysis confirmed this finding and demonstrated that Bcl2a1 mRNA expression was significantly greater in nonapoptotic than in apoptotic T lymphocytes. The RXR agonist 9-*cis*-retinoic acid also increased Bcl2a1 expression, although all-*trans*-retinoic acid and ligands for other RXR partner receptors did not. Treatment with AGN194204 and 9-*cis*-retinoic acid significantly decreased apoptosis measured by annexin V staining but did not affect expression of Bcl2 and Bcl-x_L. Bcl2a1 promoter activity was examined using a luciferase promoter construct. Both AGN194204 and 9-*cis*-retinoic acid significantly increased luciferase activity. In summary, these data demonstrate that RXR agonists increase Bcl2a1 promoter activity and increase expression of Bcl2a1 in naive T lymphocytes but do not affect Bcl2 and Bcl-x_L expression in naive T lymphocytes. Thus, this effect on Bcl2a1 expression may account for the decreased apoptosis seen in naive T lymphocytes treated with RXR agonists. *The Journal of Immunology,* **2005, 175: 7916–7929.**

Vitamin A has long been called the "the anti-infective vitamin" (1). This designation is appropriate because vitamin A deficiency impairs both innate and adaptive immunity and increases the risk of death from infectious disease (2). Vitamin A (retinol) is converted to its active metabolites all-*trans*- and 9-*cis*-retinoic acid by specific enzymes (3). Retinoic acid then regulates gene expression by binding to two nuclear receptors, the retinoic acid receptor (RAR-α),[3] -β, and -γ, also referred to as NR1B1, NR1B2, and NR1B3, respectively, and the retinoid X receptor (RXR-α), -β, and -γ, or NR2B1, NR2B2, and NR2B3, respectively (4). All-*trans*-retinoic acid binds with high affinity to RAR while 9-*cis*-retinoic acid binds with high affinity to both RAR and RXR (5, 6). RXR homodimers or heterodimers formed with RAR or other partner receptors bind to response elements in the promoter-enhancer region of target genes to regulate transcription (4).

Nuclear receptors, including RAR and RXR, play an important role in the immune system, including T lymphocyte development, function, and survival (7–11). For example, treatment of naive T lymphocytes with 9-*cis*-retinoic acid and the RXR-selective agonist AGN194204 enhance in vitro development of Th2 memory cells (12). All-*trans*-retinoic acid has similar effects (13, 14).

Apoptosis is an important regulator of the innate and adaptive immune response (15). For example, apoptosis is induced during thymic selection to minimize the development of self-reactive T lymphocytes, and protection of "appropriately" stimulated thymocytes from apoptosis helps ensure generation of a naive T lymphocyte repertoire adequate for defense against pathogenic organisms (16). In the periphery, naive T lymphocytes induce expression of the antiapoptotic proteins Bcl2a1 and Bcl-x_L following stimulation via the TCR to ensure survival during the first few days following antigenic stimulation. During the same time period, cytokines such as IL-2 and IL-4 also enhance survival by stimulating Bcl2 expression (17). In contrast, apoptosis may be induced by overstimulation of the TCR (activation-induced cell death, mediated by Fas-ligand engagement on targeted lymphocytes) and by cytokine withdrawal (death by neglect, which can be reversed by cytokines such as IL-2, IL-4, and IL-7) (18). This balance of survival and apoptosis helps maintain protective immune responses and ensures their successful termination and also protects the host from damaging autoimmune responses.

Little is known about the effect of retinoids on the survival of naive or memory T lymphocytes. However, retinoids are known to affect the survival of thymocytes, T cell hybridomas, and T lymphoma cell lines. For example, retinoic acid and RAR-selective ligands can inhibit Fas-mediated apoptosis by diminishing cell surface Fas ligand expression in T cell hybridomas activated via the TCR (19–22). This antiapoptotic effect is mediated via RAR-α/RXR heterodimers (23), whereas RAR-γ agonists can up-regulate FAS-ligand and thus facilitate apoptosis in T cell hybridomas (24)

*U.S. Department of Agriculture (USDA) Western Human Nutrition Research Center and Nutrition Department, University of California, Davis, CA, 95616; †Department of Pathology, University of California, Davis, School of Medicine, Sacramento, CA 95817; and ‡Retinoid Research, Department of Biology, Allergan, Irvine, CA 92623

Received for publication July 8, 2005. Accepted for publication September 13, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by USDA National Research Initiative Grant 97-35200-4229, USDA cooperative agreements 585306-0-233 and 58-5306-1-451, and National Institutes of Health Grant 1 R01 AI 50863.

[2] Address correspondence and reprint requests to Dr. Charles B. Stephensen, Nutrition Department, 3243 Meyer Hall, One Shields Avenue, University of California, Davis, CA 95616. E-mail address: cstephen@whnrc.usda.gov

[3] Abbreviations used in this paper: RAR, retinoic acid receptor; RXR, retinoid X receptor; PPAR, peroxisome proliferator-activated receptor; HSP70, heat shock protein 70; qRT-PCR, quantitative RT-PCR; 7-AAD, 7-aminoactinomycin D; Crabp2, cellular retinoic acid binding protein 2; DR, direct repeat; Hprt, hypoxanthine guanine phosphoribosyl transferase; VDR, vitamin D receptor; TR, thyroid hormone receptor; FXR, farnesoid X receptor; LXR, liver X receptor.

Copyright © 2005 by The American Association of Immunologists, Inc.

0022-1767/05/$02.00

as well as thymocytes (25). Thymocyte apoptosis has also been reduced by retinoic acid treatment (26). However, in T lymphoma cells, retinoic acid has been shown to induce apoptosis, although the mechanism is not known (27). To our knowledge, the effects of retinoids on apoptosis have not been examined in primary T lymphocytes.

In the present study, we have identified genes that are differentially regulated by the RXR agonist AGN194204 during primary antigenic stimulation of naive $CD4^+$ T lymphocytes from $RAG1^{-/-}$ DO11.10 TCR-transgenic mice (28). Many genes playing a role in apoptosis were identified. One of these was *Bcl2a1*, which encodes the antiapoptotic Bcl2 family member A1 (also called *Bfl-1* and *Hbpa1*) (29). Bcl2a1 is expressed in many cells of the immune system, including myeloid progenitor cells (30), macrophages (31), neutrophils (32), thymocytes (13), T lymphocytes (33), and B lymphocytes (34). In T lymphocytes, Bcl2a1 expression is induced by engagement of the TCR (17) via activation of NF-κB (17, 34, 35). Our data suggest that RXR agonists enhance survival of naive T lymphocytes by increasing *Bcl2a1* gene expression and thereby decreasing apoptosis.

Materials and Methods

Mice

$RAG1^{-/-}$ DO11.10 mice were bred in our facility. They were a gift from Dr. C. London (University of California, Davis, CA). To confirm their identity, peripheral blood from these mice was screened by two-color flow cytometric analysis using anti-CD4 Ab and the TCR clonotypic mAb KJ1–26 (36). BALB/c mice were purchased from Charles River Laboratories.

Cell lines and reagents

The Th1 and Th2 cell cultures were derived from DO11.10 mice as described (37) and were a gift of Dr. C. Weaver (University of Alabama, Birmingham, AL). The RAR and RXR plasmids used as controls in Fig. 1 were a gift from Dr. M. Satre of (University of California, Davis, CA). Abs used in cell culture were purchased from BD Pharmingen and included neutralizing rat mAbs for murine IL-4 (clone BVD40-1D11, IgG2b isotype), IL-12 (p40/p70, clone C17.8, IgG2a isotype) and IFN-γ (clone R4-6A2, clone R4-6A2). Isotype control Abs for IgG1 (clone 11010D), IgG2a (clone 11020D), and IgG2b (clone R35-38) were also used. Recombinant purified IL-4 was also purchased from BD Pharmingen. Abs for FACS analysis included PE-labeled anti-CD4 (clone L3T4; BD Pharmingen) and FITC-labeled KJ1–26 (Caltag Laboratories). The following Abs (identified by product number) were used for Western blot analysis and were purchased from Santa Cruz Biotechnology: RAR-α, SC551; RAR-β, SC552; RAR-γ, SC773; RXR-α, SC553; and RXR-γ, SC555. The Ab for RXR-β (PA1-815) was purchased from Affinity BioReagents. The secondary goat anti-rabbit Ab for Western blots was purchased from Jackson ImmunoResearch Laboratories.

The RXR-selective agonist AGN194204 (38) and other nuclear receptor agonists were diluted in cell culture-grade DMSO (Sigma-Aldrich) and stored at −70°C. Aliquots were thawed and diluted into medium immediately before addition to lymphocyte cultures. The final concentration of DMSO in the cell culture medium was 0.1%. The peroxisome proliferator-activated receptor (PPAR)-γ agonist ciglitazone was purchased from Cayman Chemical, whereas 9-*cis*-retinoic acid, all-*trans*-retinoic acid, 1α,25-dihydroxyvitamin D_3, 3,3′,5-triiodo-L-thyronine, chenodeoxycholic acid, and 22(R)-hydroxycholesterol were purchased from Sigma-Aldrich.

For reporter gene analysis, the CAT reporter plasmid A1/L-CAT, containing 2 kb of genomic DNA cloned from immediately upstream of the *Bcl2a1a* gene (39), was provided by S. Gerondakis (The Royal Melbourne Hospital, Parkville, Victoria, Australia) and cloned in a luciferase reporter plasmid (pGL3/Bcl2a1). The heat shock protein 70 (HSP70)-β-galactosidase reporter plasmid was provided by R. Modlin (University of California, Los Angeles, CA). The expression plasmid for pCMX/RXRα and pCMX were provided by D. Mangelsdorf (Southwestern Medical Center, Dallas, TX).

3T3 cells (a murine fibroblast cell line, ATCC CRL1658) and EL4 cells (a T lymphoma cell line, ATCC TIB-39) were obtained from the American Type Culture Collection. Raw 264.7 cells (a murine monocytic cell line, ATCC TIB-71) were a gift from Dr. D. Hwang (U.S. Department of Agriculture Western Human Nutrition Research Center, University of California, Davis, CA).

Cell culture

For microarray analysis, T lymphocytes were isolated and stimulated as described in two independent experiments (12). Briefly, $CD4^+$ cells were purified from $RAG1^{-/-}$ DO11.10 mice by positive selection using CD4-specific magnetic beads (Dynal Biotech). These cells were stimulated with $OVA_{323–339}$ peptide (Alpha Diagnostics International) and irradiated splenocytes from BALB/c mice as APCs. Cultures were treated with the RXR agonist AGN194204 (10 nM in 0.1% DMSO) or 0.1% DMSO (vehicle control). For RNA extraction and microarray analysis $CD4^+$/KJ1–26^+ T lymphocytes were sorted to >96% purity using a MoFlo (DakoCytomation) high-speed cell sorter.

Three independent experiments were done to confirm the microarray results and to determine whether cytokines regulating Th1/Th2 development affected expression of putative RXR-responsive genes identified in the microarray experiments. These experiments were done as just described with the following treatments: 1) AGN194204 (10 nM + 0.1% DMSO) plus neutralizing Abs for IL-4, IFN-γ, and IL-12 (5 μg/ml of each Ab); 2) AGN194204 plus isotype control Abs (5 μg/ml of each Ab); 3) vehicle control (0.1% DMSO) plus neutralizing Abs; 4) vehicle control plus isotype control Abs; and 5) IL-4 (10 ng/ml + 0.1% DMSO).

In other experiments, $CD4^+$ cells were purified from draining lymph nodes and spleens of $RAG1^{-/-}$ DO11.10 mice by positive selection using CD4-specific magnetic beads and then stimulated with plate-bound anti-CD3 (1 μg/ml; clone 145-2C11; BD Pharmingen) and anti-CD28 added to the medium (5 μg/ml; clone 37.51; BD Pharmingen). Culture conditions have been described previously (12).

The Th1 and Th2 cell cultures were stimulated every 2 wk with $OVA_{323–339}$ peptide Ag and irradiated splenocytes plus added IL-2 (50 U/ml). Culture conditions have been described previously (12).

mRNA analysis

DNA microarray analysis. Microarray analysis was done twice, using RNA from two independent experiments. RNA quality and integrity was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies) and absorbance at A260/A280. Only high-quality RNA, having a 28S/18S rRNA ratio of 1.5:2 and an A260/280 ratio of 1.8:2, was used for further experimentation. RNA was converted to double-stranded cDNA and to biotin-labeled cRNA by in vitro transcription labeling with a HighYield BioArray RNA Transcript Labeling kit (Enzo Biochem). The quality of in vitro transcription and fragmentation products was assessed using the Agilent 2100 Bioanalyzer.

We used the U74Av2 murine oligonucleotide arrays (Affymetrix) that contain 12,488 probes representing 5,890 genes. Fifteen micrograms of fragmented, biotin-labeled cRNA was hybridized at 45°C overnight as defined in the Affymetrix7 expression analysis protocol. The hybridization buffer contained 100 mM MES, 1 M NaCl, 20 mM EDTA, 0.01% Tween 20, four eukaryotic hybridization controls (1.5 pM BioB, 5 pM BioC, 25 pM BioD, and 100 pM cre), 0.1 mg/ml herring sperm DNA (Promega), and 0.5 mg/ml acetylated BSA. After hybridization, the arrays were washed and stained with an Affymetrix fluidic station following the Ab Amplification Washing and Staining Protocol (Affymetrix). Hybridization was detected with streptavidin-PE and a confocal laser scanner (Affymetrix).

PCR analysis. Qualitative PCR analysis to confirm RAR and RXR expression, and quantitative PCR (qRT-PCR) analysis to compare levels of gene expression were done as described previously (12). Primers are described in Table I and were developed using GCG software (Genetics Computer Group). Primers used for Bcl2 for were purchased from BioSource International. qRT-PCR was performed using a LightCycler rapid thermal cycler system with SYBR Green I dye (Roche Diagnostic Systems). RNA quality for qRT-PCR analysis was assessed as described for microarray analysis. Melting curve analysis was used to confirm specificity of the products. Products were cloned and sequenced during assay development to confirm specificity.

Apoptosis analysis

Apoptotic cells were identified using flow cytometric analysis of cells stained with PE-labeled annexin V to identify apoptotic cells and 7-aminoactinomycin D (7-AAD) to label permeable (dead) cells (BD Pharmingen), and staining was done according to the manufacturer's instructions using a FACSCalibur flow cytometer (BD Pharmingen). Apoptosis assays were performed on CD4-selected naive T lymphocytes stimulated with anti-CD3 and anti-CD28 Ab. In one experiment, these reagents were used to sort viable, nonapoptotic cells (annexin V^-/7-AAD^-) from viable, apoptotic cells (annexin V^+/7-AAD^-) using a MoFlo high-speed cell sorter.

Table I. *Sequence of primers used for RT-PCR and quantitative real-time PCR analysis*

Target Gene	Primer Sequence	Genbank Accession No.	Primer Location (from 5′ end of cDNA)
RAR-α	5′-TCATGAAGTGTGACGTTGACATCCGT-3′	M60909	1031–1053
	5′-TTGGCGAAGGCAAAGAC-3′		1345–1329
RAR-β	5′-AAATACACCACGAATTCCAG-3′	S56660	956–975
	5′-TCATCCATTTCCAAAGGC-3′		1305–1288
RAR-γ	5′-CATCACCAAGGTCAGCAAAG-3′	X15848	882–901
	5′-TCTCGGTGTCATCCATCTCC-3′		1297–1278
RXR-α	5′-AACCCCAGCTCACCAAATGACC-3′	X66223	961–982
	5′-AACAGGACAATGGCTCGCAGG-3′		1319–1299
RXR-β	5′-GCTCCTCATTGCGTCCTTCTCC-3′	X66224	873–894
	5′-CGTAACAGCAGCTTGGCAAACC-3′		1214–1193
RXR-γ	5′-CCGCTGCCAGTACTGTCG-3′	X66225	689–706
	5′-ACCTGGTCCTCCAAGGTGAG-3′		1018–999
β-actin	5′-TCATGAAGTGTGACGTTGACATCCGT-3′	NM007393	925–950
	5′-CCTAGAAGCATTTGCGGTGCACGATG-3′		1209–1184
Crabp2	5′-GGAGATTAACTTCAAGATCGGGGA-3′	M35523	301–324
	5′-GCTAGTTTGTAAGATGGACGTGGG-3′		621–597
Bcl2a1	5′-CTTCAGTATGTGCTACAGGTACCCG-3′	U23779	180–204
	5′-TGGAAACTTGTTTGTAAGCACGTACAT-3′		490–465
Bcl-x_L	5′-AGAAGAAACTGAAGCAGAG-3′	NM009743.2	216–234
	5′-TCCGACTCACCAATACCTG-3′		580–598
Hprt	5′-GTTGGATACAGGCCAGACTTTGTTG-3′	NM013556	601–625
	5′-GAGGGTAGGCTGGCCTATAGGCT-3′		952–930

To determine the purity after sorting, some cells were collected into Annexin V Binding buffer (BD Pharmingen) to retain binding of annexin V. Sorted cells were >90% pure.

Western blots

Cells were washed in cold PBS containing 0.5 mM PMSF before protein extraction. Whole-cell protein extracts were prepared as follows: washed cells were resuspended in lysis buffer (1% Nonidet P-40, 30 mM Tris-HCl (pH 7.5), 0.5 mM EDTA (pH 8.0), 150 mM NaCl, 10% glycerol, 0.5 mM PMSF, and 1/500 dilution of protease inhibitor solution) and were incubated for 5 min on ice. The protease inhibitor solution consisted of one complete protease inhibitor tablet (Roche Diagnostic Systems) dissolved in 250 μl of water. A total of 5 M NaCl was added to a final concentration of 400 mM, and cells were incubated for an additional 10 min on ice. Cells were then passed through a Qiashredder (Qiagen) by centrifugation for 10 min at 20,800 $\times$ *g* at 4°C. Nuclear extracts were prepared as follows: washed cells were resuspended in 100 μl of solution A (0.5% Nonidet P-40, 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, and 10 mM KCl) per million cells and incubated for 5 min on ice. Samples were then pelleted by centrifugation at 6800 $\times$ *g* at 4°C for 5 min. The pellet was resuspended in 25 μl of solution B (20 mM HEPES (pH 7.9), 25% glycerol, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA (pH 8), 0.2 mM PMSF, 0.5 mM DTT, and 1/500 dilution of protease inhibitor solution) per million cells. Samples were frozen and thawed three times and passed through a Qiashredder as described above.

Proteins were resolved by SDS-PAGE on 4–12% gradient gels (Novex) and transferred to nitrocellulose (Invitrogen Life Technologies). Blots were blocked using 10% skim milk powder in PBS containing 0.1% polyoxyethylenesorbitan monolaurate (Sigma-Aldrich) (PBST) overnight at 4°C. Blots were washed three times with PBST and were blocked again in 5% goat serum (Jackson ImmunoResearch Laboratories) in PBST overnight at room temperature. Each blot was then probed using appropriate Abs diluted at 1/500 at room temperature for 1.5 h and washed three times with PBST. After washing, the secondary Ab diluted at 1/20,000 was added and incubation lasted another 1.5 h at room temperature. Finally, the blots were washed three times with PBST and developed using the ECL kit (Amersham Biosciences).

Reporter gene analysis

Cell lines were maintained in DMEM (Invitrogen Life Technologies) supplemented with 10% FBS (FCS) (Invitrogen Life Technologies) and 1$\times$ Antibioticum/Antimycoticum (Invitrogen Life Technologies) at 37°C in a 5% CO_2/air environment. Cells for reporter assays were seeded in 24-well plates at a density of 1.5–2.0 $\times$ 10^5 cells/well 1 day prior transient cotransfections. Before transfection, the growth medium was replaced with DMEM, supplemented with 10% charcoal/dextran-treated FBS. Per well, 3T3 cells were transfected with 500 ng of pCMX or pCMX/RXRα, 400 ng of pGL3-basic/Bcl2a1, and 200 ng of HSP70-β-galactosidase reporter plasmid using LipofectAMINE 2000 (Invitrogen Life Technologies), following the manufacturer's instructions; Raw 264.2 cells were transfected in the same way with 500 ng of pCMX or pCMX/RXRα, 800 ng of pGL3-basic/Bcl2a1, and 400 ng of HSP70-β-galactosidase reporter plasmid. Twenty-four hours after transfections, the cells were treated with DMSO, 9-*cis*-RA, or AGN 194204 at 1 μM or at a range from 1 nM to 10 μM. Twenty-four hours after treatment, the cells were harvested in reporter lysis buffer (Promega). Luciferase and β-galactosidase enzyme activities were determined using Luciferase Assay System and β-galactosidase Enzyme Systems (Promega) following the manufacturer's instructions. Luciferase activity was determined by a TD-20/20 Luminometer (Turner Designs); β-galactosidase activity was assayed using an EL_X800 Universal Microplate Reader (Bio-Tek Instruments).

Statistical analysis

Microarray analysis. Microarray Suite 5.0 (Affymetrix) was used to determine the probe intensities and to compare expression among different arrays; the average intensity for each array was normalized by scaling to a target median intensity value of 125. The gene expression values were log transformed (log base 2). Genes were ranked on *t* test scores, *p* values ($p < 0.05$), and fold changes computed as actual expression values. A particular transcript was considered significantly differentially expressed between the groups if it had a fold change > 1.29 (RXR agonist vs vehicle control or vice versa) and a value of $p < 0.05$, and this finding was concordant in the two independent experiments. The cutoff of >1.29 was selected because previous quantitative RT-PCR analysis (12) showed that changes of this magnitude in Th2-enhancing genes (*IL-4* and *GATA-3*) were both statistically significant and predicted later development of a Th2 phenotype. The annotation of the differentially expressed genes was performed using information from the Affymetrix and National Center for Biotechnology Information EntrezGene web sites.

Other statistical analysis. Statistical analysis was performed using SigmaStat for Windows 2.03 or 3.1 (SPSS). Multiple comparisons of RNA levels determined by RT-PCR and percentage of apoptotic cells were made using several methods. Paired Student's *t* tests were used to control for experiment or time within an experiment when comparing retinoid treatments. One-way ANOVA was used to compare retinoid treatments within a particular experiment. Two-way ANOVA was used to compare retinoid treatments while controlling for the experiment when results from multiple experiments were pooled for analysis. Three-way ANOVA was also used to control for experiment when comparing two treatment variables, such as retinoid treatment and partner ligand treatment, or cytokine treatment and retinoid treatment. Two-way repeated-measure ANOVA was used to compare retinoid treatments when controlling for time and experiment. Duplicates to quadruplicate measurements were made within each experiment,

and experiments were repeated at least three times. A value of $p < 0.05$ using a two-tailed test was considered statistically significant.

Results

Expression of RAR and RXR in CD4$^+$ T lymphocytes

Treatment of naive CD4$^+$ T lymphocytes from DO11.10 mice with RAR and RXR agonists affects Th1/Th2 memory cell development (12, 40, 41). Therefore, we wished to determine which RAR and RXR receptors are expressed in naive or memory CD4$^+$ T lymphocytes from these mice. Using qualitative PCR analysis of cDNA, we detected a single band of appropriate size for RAR-α, RAR-γ, RXR-α, and RXR-β in naive DO11.10 CD4$^+$ T lymphocytes (Fig. 1, *top panel*) and in Th1 and Th2 cells derived from DO11.10 mice (data not shown). The cDNA sequences of these products were essentially identical to the published sequences for these receptors. Western blot analysis of nuclear extracts found that RAR-α, RXR-α, and RXR-β but not RAR-β, RAR-γ, or RXR-γ were present in naive CD4$^+$ T lymphocytes as well as in the Th1 and Th2 cultures (Fig. 1, *bottom panel*). Although RAR-γ mRNA was found at levels similar to the other receptors, protein was not seen in either whole cell or nuclear extracts.

RAR and RXR expression following primary stimulation of naive CD4$^+$ T lymphocytes

We previously reported that treatment of naive DO11.10 T lymphocytes with the RXR agonist AGN194204 increases Th2 development and early expression of Th2-promoting genes (12). To determine whether expression of RAR and RXR were also affected by this treatment, samples from these experiments were examined by qRT-PCR. RNA levels for both RAR-α and RAR-γ decreased after antigenic stimulation (Fig. 2). No differences among the three treatments (RXR agonist, vehicle control, IL-4) were seen for RAR-γ, but RAR-α RNA levels were higher in the IL-4 treatment than in the vehicle control (Fig. 2). Although both RXR-α and RXR-β mRNA levels were higher in the IL-4 treatment than in the vehicle control after stimulation (Fig. 2), treatment with the RXR agonist did not have a consistent effect on RAR or RXR expression.

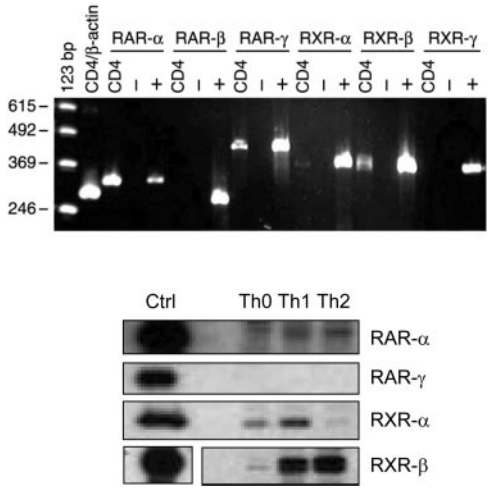

FIGURE 1. Qualitative PCR and Western blot analysis of RAR and RXR expression in naive DO11.10 T lymphocytes and in Th1 and Th2 DO11.10 cells. Naive CD4$^+$ cells were purified from lymph nodes and spleens of DO11.10 mice by positive selection using magnetic beads. Cultured Th1 and Th2 cells were purified away from irradiated APCs by Ficoll density gradient centrifugation 7–14 days after stimulation with $OVA_{323–339}$ peptide Ag. *Top panel*, Expression of RAR and RXR mRNA was assessed by qualitative PCR in total RNA extracted from naive T lymphocytes (CD4). Plasmid DNA from each gene served as a positive control. RT-PCR mixes without template served as negative controls for each reaction (−). *Bottom panel*, Nuclear extracts from naive (Th0) DO11.10 T lymphocytes and DO11.10 Th1 and Th2 cells (Th1, Th2) were analyzed by Western blot analysis. Cell lysates containing the appropriate baculovirus-expressed mouse receptor protein were included as a positive control (Ctrl). The protein content of each lane for RAR-α, RAR-γ, and RXR-α were Th0 (3 μg), Th1 (0.5 μg), and Th2 (1.5 μg); for RXR-β, 15 μg were used for each cell type.

DNA microarray analysis following primary stimulation of naive CD4$^+$ T lymphocytes

Comparison of cultures treated with the RXR agonist AGN194204 (100 nM) or vehicle control (0.1% DMSO) revealed differential expression of 128 genes (Table II), 108 of which have a known function. When examined by functional category, regulation of cell growth and apoptosis encompassed the greatest number of genes (16 of 108 = 15%), followed by regulation of transcription (10 of 108 = 9%). Other functional groups (Table II) were represented at <7%/group.

Several genes known to be directly regulated by RXR homo- or heterodimers were identified. For example, mRNA levels of the stearoyl-CoA desaturase 1 (*Scd1*) gene are increased by both RAR and RXR agonists (42). In this analysis, the mean fold-increases for Scd1 RNA was 17.1 (Table II). The cellular retinoic acid binding protein 2 (*Crabp2*) gene, which contains prototypical direct repeat (DR)1 and DR2 response elements and is up-regulated by both RAR and RXR agonists (43), showed the greatest increase of all genes 24-fold (Table II and Fig. 3). These observations confirm that genes regulated by RAR and RXR can be identified from these experiments.

This microarray analysis also confirmed our previous observations (12) that RNA levels for Th2-enhancing genes are increased by treatment with the RXR agonist. In this analysis, these included genes for *IL-4* (1.9-fold), *GATA3* (1.5-fold), *IL-4Rα-chain* (1.4-fold), and growth factor independent (*GFI-1*) (1.3-fold), although mRNA for IL-13 was decreased (−2.8-fold). mRNA levels for genes involved in Th1 growth and development were decreased by treatment with the RXR agonist, including IL-2 (−1.4-fold), IL-2Rα-chain (−1.4-fold), and IL-12Rβ1- (−1.4-fold) and IL-12R-β2 chains (−1.8-fold).

The high percentage of cell growth and apoptosis genes identified in this analysis was of interest for two reasons. First, retinoids are known to regulate apoptosis in T lymphocytes, thymocytes, and T lymphoma cells (7, 21, 44). Second, in addition to promoting Th2 development, treatment with the RXR agonist AGN194204 during primary antigenic stimulation reproducibly caused a greater accumulation of viable cells after 1 wk of culture than did treatment with the vehicle control. In eight independent experiments the number of viable cells (±SD) in the RXR agonist (10^{-7} or 10^{-8} M), IL-4 (10 ng/ml) and vehicle control (0.1% DMSO) treatments were 3.80 ± 2.78, 3.73 ± 1.50, and 2.36 ± 1.91 × 10^6 cells/ml, respectively. Cell counts in the RXR and IL-4 treatment groups were both significantly greater than the counts in the vehicle control ($p = 0.032$ and $p = 0.024$, respectively, by paired Student's t test), while the RXR agonist and IL-4 treatments did not differ from one another ($p = 0.92$). The mean fold increases for the RXR and IL-4 treatments were 2.24 and 2.37, respectively.

Because treatment with the RXR agonist increases cell number, we examined differential expression of cell growth and apoptosis genes in more detail. The expression of Bcl2a1 mRNA (Table II; Fig. 3) was significantly greater in cultures treated with AGN194204 than in the vehicle control cultures (~2-fold). Bcl2a1

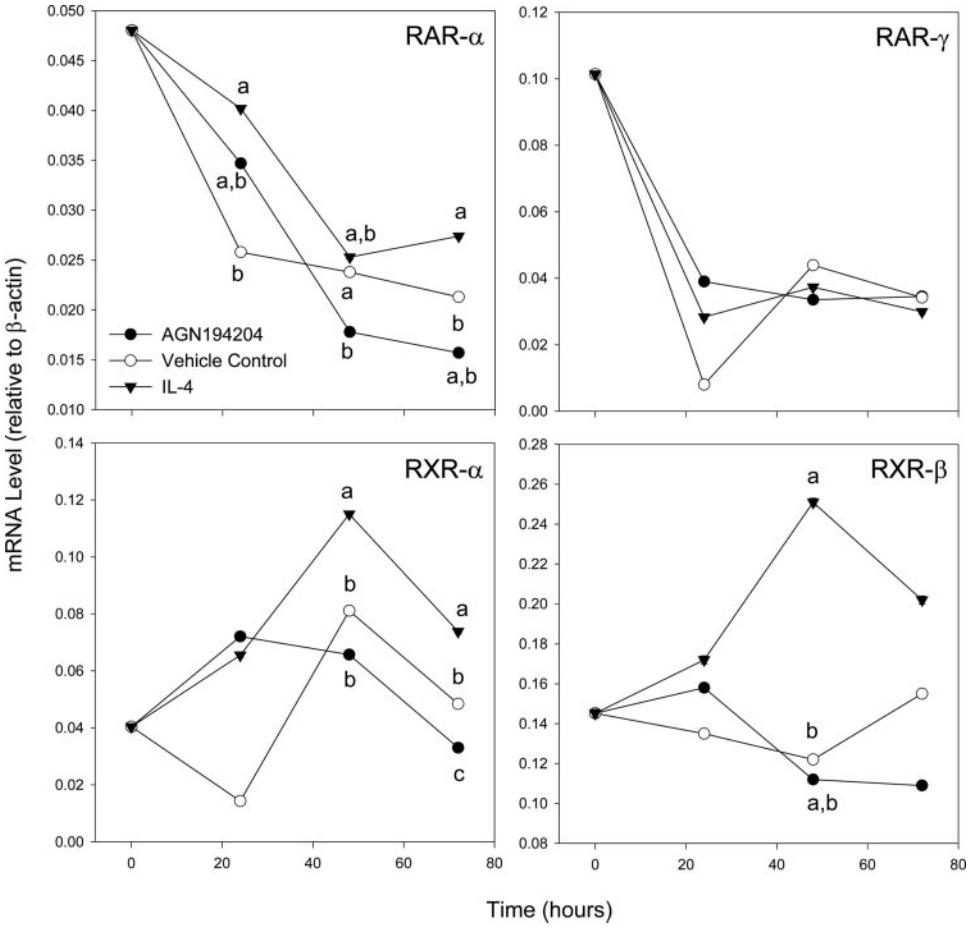

FIGURE 2. qRT-PCR analysis of RAR and RXR expression in naive DO11.10 T lymphocytes sorted to >96% purity 64 h following primary antigenic stimulation with irradiated splenocytes and $OVA_{323–339}$ peptide Ag. Cultures were treated at the time of stimulation with the RXR agonist AGN194204 (10 nM in 0.1% DMSO), IL-4 (10 ng/ml in 0.1% DMSO), or vehicle control (0.1% DMSO). Data are mean values of duplicate measurements from two independent experiments. Significant differences among the treatment means at each time point were determined by two-way ANOVA (analyzed by treatment group and experiment using all pairwise comparisons and Bonferroni's correction). Means that are significantly different from one another at the same time point do not share the same superscript letter (a, b, and c). Expression of other mRNAs has been published previously from these samples (12).

also stood out because of the high signal intensity for the two Bcl2a1 probe sets (third and fourth highest of all differentially expressed genes). These two probe sets represent the genes *Bcl2a1b* and *Bcl2a1d*, two members of a family of four genes (also including *Bcl2a1a* and *Bcl2a1c*, although the latter gene is not expressed) that code for the B cell leukemia/lymphoma 2-related protein A1 (45). These genes have high sequence identity and are Bcl2 family members. As with Bcl2, the Bcl2a1 protein acts at the mitochondrial level to prevent apoptosis and is known to be expressed and protective against apoptosis in naive T lymphocytes undergoing primary Ag stimulation (33).

RXR-stimulated increase in Bcl2a1 is independent of IL-4, IL-12, and IFN-γ stimulation

To confirm the microarray results, we examined *Bcl2a1* gene expression by qRT-PCR using primers that detect all Bcl2a1 family members. In addition, because treatment with AGN194204 and other retinoids affect Th1/Th2 cytokine production (12), neutralizing Abs for IL-4, IL-12, and IFN-γ were included in some cultures to determine whether RXR-mediated increases in Bcl2a1 expression were independent of effects on these cytokines (Fig. 4). The mean Bcl2a1 mRNA levels in the AGN194204 treatment groups was greater than in the vehicle control groups either in the presence ($p < 0.001$, fold increase = 1.83) or in the absence ($p < 0.001$, fold increase = 2.38) of the cytokine-neutralizing Abs (Fig. 4). Treatment of cultures with IL-4 did not enhance Bcl2a1 expression (Fig. 4). These results are consistent with the findings from the microarray studies and also indicate that RXR-induced increases in Bcl2a1 mRNA occur independent of IL-4, IL-12, and IFN-γ stimulation.

Crabp2 mRNA levels were also analyzed in this experiment to confirm transcriptional activity of the RXR agonist (Fig. 4). Mean Crabp2 RNA levels following treatment were also higher compared with the levels of DMSO-treated cells either in the presence ($p < 0.001$, fold increase = 4.11) or in the absence ($p < 0.001$; fold increase = 5.58) of the cytokine-neutralizing Abs. Treatment with the RXR agonist produced no consistent, statistically significant differences in hypoxanthine guanine phosphoribosyl transferase (Hprt) mRNA levels either in the presence or absence of neutralizing Ab (Fig. 4). This result was also consistent with our microarray studies (Fig. 3).

*Time course of Bcl2a1 RNA expression: effect of AGN194204 and 9-*cis*-retinoic acid*

The expression of Bcl2a1 is induced in T lymphocytes by stimulation via the TCR complex. Expression peaks before 24 h and then decreases by 48 h (17, 46). Purified $CD4^+$ DO11.10 T lymphocytes were stimulated with anti-CD3 plus anti-CD28 Ab in the presence of AGN194204, the natural RXR agonist 9-*cis*-retinoic acid (100 nM), or vehicle control (0.1% DMSO). Bcl2a1 mRNA levels were greatest at 20 h in cells treated with vehicle only (Fig. 5). The pattern was similar following retinoid treatments. AGN194204 significantly increased Bcl2a1 expression at both 20 h (41% higher) and 44 h (38% higher). The effect of 9-*cis*-retinoic acid on Bcl2a1 expression was similar to that of AGN194204 (Fig. 5), although the magnitude of the effect was less. At 20 and 44 h following 9-*cis*-retinoic acid treatment, the increases in Bcl2a1 expression over the vehicle control were 23 and 42%, respectively ($p = 0.027$, paired t test). Crabp2 RNA expression was examined as a positive control for the retinoid

Table II. *Genes differentially expressed in sorted (>96% purity) naive DO11.10 T lymphocytes 64 h after antigenic stimulation (using irradiated splenocytes plus $OVA_{323–339}$ peptide) and treatment with the RXR agonist AGN194204 (10 nM) or vehicle control (0.1% DMSO)*

Gene Designation and Name[a]	Process (function)[a]	Chr[a]	Fold Change[b]	RXR Agonist[b]	Vehicle Control[b]	Gene (GenBank)	Probe Set (Affymetrix)
Crabp2, cellular retinoic acid binding protein II	Transport (lipid binding)	2	24.50	698	33	M35523	100127_at
Spp1, secreted phosphoprotein 1	Cell adhesion (cytokine activity)	5	19.03	146	17	X13986	97519_at
Scd1, stearoyl-CoA desaturase 1	Fatty acid biosynthesis	19	17.10	63	14	M21285	94057_g_at
Adam 19, a disintegrin and metalloproteinase domain 19	Proteolysis and peptidolysis	11	5.87	161	27	AA726223	103554_at
Camk2d, calcium/calmodulin-dependent protein kinase II, δ	G_1-S transition, autophosphorylation, Ca transport	3	4.26	36	13	AF059029	93214_at
Phlda1, pleckstrin homology-like domain, family A, member 1	Apoptosis, FasL biosynthesis	10	3.38	281	85	U44088	160829_at
Plac8, placenta-specific 8		5	2.96	571	201	AA790307	98092_at
Pdcd4, programmed cell death 4	Apoptosis (DNA topoisomerase)	19	2.55	396	156	D86344	103029_at
Scd2, stearoyl-CoA desaturase 2	Fatty acid biosynthesis	19	2.48	1361	552	M26270	95758_at
Stra6, stimulated by retinoic acid gene 6		9	2.45	177	73	AF062476	102258_at
Dscr1l2, down syndrome critical region gene 1-like 2	(calcium-mediated signaling)	4	2.37	194	89	AI847661	102374_at
A630084M22Rik Riken cDNA	Intracellular protein transport, small GTPase mediated signal transduction	1	2.26	219	99	AI846023	92185_at
IL-4, interleukin 4	B cell activation, immune response (cytokine, growth factor)	11	2.23	72	38	X03532	92283_s_at
H1f0, H1 histone family, member 0	Chromosome organization, biogenesis; nucleosome assembly	15	2.10	859	411	M29260	95419_at
Adfp, adipose differentiation related protein		4	2.04	245	122	M93275	98589_at
Bcl2a1d, B cell leukemia-lymphoma 2 related protein A1d	Apoptosis		1.99	2197	1104	U23781	93869_s_at
Sema4b, sema domain, Ig, transmembrane (TM) and short cytoplasmic domain, (semaphorin) 4B	Development, neurogenesis (receptor)	7	1.95	122	61	AA266467	95387_f_at
Scd1, stearoyl-CoA desaturase 1	Fatty acid biosynthesis	19	1.95	99	52	M21285	94056_at
Nrip1, nuclear receptor interacting protein 1	Regulation of transcription from Pol II promoter (corepressor)	16	1.95	186	96	AF053062	103288_at
Cotl1, coactosin-like 1 (dictyostelium)	(actin binding, protein binding)	8	1.94	509	307	AI837006	95466_at
IL-4, interleukin 4	B cell activation, immune response (cytokine, growth factor)	11	1.91	129	73	M25892	AFFX-MurIL4_at
Hic1, hypermethylated in cancer 1	Development, regulation of transcription, DNA-dependent	11	1.84	304	167	AW048074	104462_at
Itgb2, integrin β 2	Cell and cell-matrix adhesion, extravasation, integrin-midated signaling	10	1.84	286	156	M31039	102353_at
Gp49a, glycoprotein 49 A		10	1.82	68	37	M65027	100325_at
Bcl2a1b B cell leukemia-lymphoma 2-related protein A1b	Apoptosis	9	1.81	2299	1272	U23778	102914_s_at
Acaa2, acetyl-CoA acyltransferase 2 (mitochondrial 3-oxoacyl-CoA thiolase)	Fatty acid metabolism	18	1.75	1174	678	AI849271	95064_at
Pros1, protein S (α)	Blood coagulation (Ca binding)	16	1.75	100	57	L27439	104728_at
Gzmb, granzyme B	Apoptosis, cytolysis (endopeptidase)	14	1.74	305	188	M12302	102877_at
Itm2a, integral membrane protein 2A		X	1.73	221	128	L38971	93511_at
Npc2, Niemann Pick type C2		12	1.73	2353	1362	AB021289	160344_at
Fnbp1, formin binding protein 1	(protein binding)	2	1.71	142	84	AW046460	103638_at
IL-4, interleukin 4	B cell activation, immune response (cytokine, growth factor)	11	1.66	143	87	AA967539	92286_g_at
Xbp1, X-box binding protein 1	Regulation of transcription, DNA-dependent	11	1.61	299	188	AW123880	94821_at
Cpt1a, carnitine palmitoyltransferase 1a, liver	Fatty acid metabolism, transport	19	1.59	408	256	AF017175	93320_at
3732413l11Rik Riken cDNA		11	1.57	235	150	AI047107	96104_at
Itgb7, integrin β 7	Cell and cell-matrix adhesion, extravasation, integrin-medated signaling	15	1.56	326	209	M68903	100906_at
Cst3, cystatin C	(cysteine protease inhibitor activity)	2	1.56	838	540	U10098	99586_at
Eno3, enolase 3, beta muscle	Glycolysis	11	1.55	450	303	X61600	96344_at
Penk1, preproenkephalin 1	Behavioral fear response, perception of pain (cytokine)	4	1.53	3283	2159	M55181	94516_f_at
Igh-6, Ig H chain 6 (H chain of IgM)	B cell proliferation, positive regulation (antigen binding)	12	1.51	193	129	V00821	93584_at
Bnip3l, BCL2/adenovirus E1B 19 kDa-interacting protein 3-like	Apoptosis	14	1.51	231	153	AF067395	96255_at
Wbp5, WW domain binding protein 5		X	1.50	210	141	U92454	100522_s_at
Rgs10, regulator of G-protein signaling 10	Signal transduction	7	1.49	311	208	AI847399	160629_at
Ldb3, LIM domain binding 3	Intracellular signaling cascade	14	1.49	529	352	AF053367	100554_at
Atp1a1, ATPase, Na^+/K^+ transporting, α 1 polypeptide	Ion transport, K and Na, metabolism (ATPase activity)	3	1.49	1247	839	AW123952	93797_g_at
Gata3, GATA binding protein 3	Cell fate determination, regulation of transcription (DNA-dependent)	2	1.47	1029	700	X55123	100924_at
Nsmaf, neutral sphingomyelinase (N-SMase) activation associated factor	(death receptor binding)	4	1.45	116	80	AF013632	160826_at

(*Table continues*)

Table II. *Continued*

Gene Designation and Name[a]	Process (function)[a]	Chr[a]	Fold Change[b]	RXR Agonist[b]	Vehicle Control[b]	Gene (GenBank)	Probe Set (Affymetrix)
Cyb5, cytochrome b-5	Fatty acid metabolism (stearoyl-CoA 9-desaturase activity), electron transport	18	1.45	458	322	AI854779	98533_at
Cd3e, CD3 antigen, ε polypeptide	Cell surface receptor linked signal transduction	9	1.45	900	623	M23376	102971_at
6330442E10Rik RIKEN cDNA		12	1.44	181	126	AI844839	103424_at
Dgka, diacylglycerol kinase, α	Intracellular signaling cascade, protein kinase C activation	10	1.44	450	313	AF085219	103596_at
Ccng2, cyclin G2	Cell cycle, cytokinesis, mitosis (cyclin dependent protein kinase regulator)	5	1.44	116	82	U95826	98478_at
Lamp1, lysosomal membrane glycoprotein 1		8	1.43	1353	947	M32015	160089_at
2610016F04Rik RIKEN cDNA	Regulation of transcription, DNA-dependent	14	1.43	223	157	AA867655	98894_at
IL-4ra, interleukin 4 receptor, α	Cell surface receptor linked signal transduction	7	1.42	641	455	M27960	102021_at
Foxp1, forkhead box P1	Regulation of transcription (repressor), negative, DNA-dependent	6	1.42	203	143	AA833293	104415_at
Atp1a1, ATPase, Na^+/K^+ transporting, α1 polypeptide	Ion transport, K and Na, metabolism (ATPase activity)	3	1.42	1699	1198	AI839988	93798_at
cDNA			1.41	167	119	AI098965	92233_at
Lrmp, lymphoid-restricted membrane protein		6	1.41	436	309	U10484	101835_at
Elovl5, ELOVL family member 5, elongation of long chain fatty acids (yeast)		9	1.41	602	428	AI852098	93496_at
Fdx1, ferredoxin 1	Electron transport	9	1.40	386	281	L29123	92587_at
Csrp1, cysteine and glycine-rich protein 1	Actin cytoskeleton organization and biogenesis	1	1.40	440	317	D88793	92608_at
Hmgb3, high mobility group box 3	DNA packaging, regulation of transcription, DNA-dependent	X	1.37	1500	1092	AF022465	98038_at
Csrp1, cysteine and glycine-rich protein 1	Actin cytoskeletal organization and biogenesis	1	1.37	426	312	AI837625	160065_s_at
cDNA			1.31	204	156	AI451153	101884_at
2510027N19Rik RIKEN cDNA	(intracellular signaling cascade)	7	1.31	449	343	AI838320	100057_at
Gfi1, growth factor independent 1	Regulation of transcription, DNA-dependent	5	1.31	305	236	U58972	103259_at
Anxa11, annexin A11	(calcium-dependent phosphatidylethanolamine binding)	14	1.31	363	277	U65986	102815_at
Atf3, activating transcription factor 3	Gluconeogenesis, regulation of transcription (DNA-dependent)	1	−1.32	127	168	U19118	104156_r_at
Traf4, TNFR-associated factor 4	Apoptosis, development (zinc ion binding)	11	−1.32	195	268	X92346	100005_at
C330018J07Rik RIKEN cDNA		16	−1.32	208	275	AI839212	160676_at
Aebp2, AE binding protein 2	Regulation of transcription, DNA-dependent	6	−1.32	307	407	AW120832	94869_at
Ezh2, enhancer of zeste homolog 2 (*Drosophila*)	Regulation of transcription, DNA-dependent; histone methylation	6	−1.34	507	680	U52951	99917_at
IL-2ra, interleukin 2 receptor, α chain	T cell homeostasis, cell surface receptor linked signal transduction	2	−1.35	217	307	M26271	101917_at
Stat5b, signal transducer and activator of transcription 5B	Regulation of transcription, DNA-dependent; signal transduction	11	−1.36	417	567	AJ237939	100423_f_at
Clic4, chloride intracellular channel 4 (mitochondrial)	Ion transport, Cl (voltage-gated ion channel)	4	−1.36	327	444	AI845237	94255_g_at
Epb4.1, erythrocyte protein band 4.1	Actin cytoskeleton organization and biogenesis	4	−1.36	140	190	L00919	160379_at
IL-2rb1, interleukin 12 receptor, β1	Cell surface receptor linked signal transduction	8	−1.37	77	107	U23922	98240_at
Man1a, mannosidase 1, α	Carbohydrate metabolism, N-linked glycosylation	10	−1.37	196	270	AI021125	160579_at
Gpr65, G protein-coupled receptor 65	G protein-coupled receptor protein signaling pathway	12	−1.37	390	536	U39827	96553_at
Mus, musculus similar to serine protease OMI (LOC384447), mRNA	(trypsin;trypsin activity;7.1e-19)		−1.38	209	289	AW047978	104102_at
IL-2, interleukin 2	Cell proliferation, immune response (cytokine, growth factor)	3	−1.39	1863	2572	M16762	AFFX-MurIL2_at
Cd24a, CD24a Ag	Defense response	10	−1.41	150	212	M58661	100600_at
Slc3a2, solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	Carbohydrate metabolism	19	−1.41	718	1017	X14309	99133_at
GAPDH, glyceraldehyde-3-phosphate dehydrogenase	Glycolysis	6	−1.41	256	362	D50430	98984_f_at
Bzw2, basic leucine zipper and W2 domains 2	Regulation of translational initiation	12	−1.44	916	1316	AW060951	95462_at
Eed, embryonic ectoderm development	Imprinting (protein binding)	7	−1.44	233	335	U78103	103342_at
Lgals1, lectin, galactose binding, soluble 1	Cell adhesion, heterophilic	15	−1.45	2464	3578	X15986	99669_at
cDNA	(Ribosomal_S18;structural constituent of ribosome;4.3e-07)		−1.46	430	625	AI846849	95159_at
Csf2, colony-stimulating factor 2 (granulocyte-macrophage)	Cytokine and chemokine mediated signaling pathway	11	−1.47	186	287	X03020	92948_at
Furin, furin (paired basic amino acid cleaving enzyme)	Proteolysis (serine-type endopeptidase)	7	−1.47	494	729	X54056	100515_at

(*Table continues*)

Table II. *Continued*

Gene Designation and Name[a]	Process (function)[a]	Chr[a]	Fold Change[b]	RXR Agonist[b]	Vehicle Control[b]	Gene (GenBank)	Probe Set (Affymetrix)
Cd6, CD6 Ag	Cell adhesion (scavenger receptor)	19	−1.48	325	481	U12434	92204_at
Cd98, CD98 Ag	Defense response	19	−1.48	667	986	AB017189	104221_at
Ifi47, interferon γ-inducible protein	Defense response (GTP-binding)	11	−1.48	116	172	M63630	104750_at
Anxa2, annexin A2	(calcium ion binding, calcium-dependent phospholipid binding)	9	−1.50	342	513	M14044	100569_at
Pim1, proviral integration site 1	Cell growth, maintenance (serine/threonine kinase)	17	−1.53	535	814	AA764261	104533_at
Aim1, absent in melanoma 1		10	−1.55	260	404	AA711704	103443_at
Scin, scinderin	(actin binding)	12	−1.57	59	92	U04354	103715_at
Clic4, chloride intracellular channel 4 (mitochondrial)	Ion transport, Cl (voltage-gated ion channel)	4	−1.57	498	786	AI849533	94256_at
Hk2, hexokinase 2	Glycolysis	6	−1.60	481	783	Y11666	94375_at
Gadd45b growth arrest and DNA damage-inducible 45β	Apoptosis, cell differentiation, regulation of protein kinase activity	10	−1.60	143	226	X54149	102779_at
Tap1, transporter 1, ATP-binding cassette, subfamily B (MDR/TAP)	Immune response, oligopeptide transport	17	−1.61	483	778	U60020	103035_at
Ccl3, chemokine (C-C motif) ligand 3	Chemotaxis	11	−1.62	226	368	J04491	102424_at
Emp3, epithelial membrane protein 3	Cell growth	7	−1.62	462	751	U87948	93593_f_at
Emp1 epithelial membrane protein 1	Cell growth	6	−1.68	33	55	X98471	97426_at
cDNA			−1.71	399	683	AV109962	162482_at
IL-2rb interleukin 2 receptor, β chain	Cell surface receptor linked signal transduction	15	−1.75	78	136	M28052	100764_at
cDNA			−1.75	681	1195	AI846118	98910_at
5830443C21Rik RIKEN cDNA		19	−1.77	77	142	AA919750	103508_at
Irf1, interferon regulatory factor 1	Regulation of transcription (DNA binding), immune response	11	−1.78	161	284	M21065	102401_at
Cd6, CD6 Ag	Cell adhesion (scavenger receptor)	19	−1.79	544	975	U37543	92203_s_at
Prnp, prion protein	Cu homeostasis, nucleic acid metabolism, response to oxidative stress	2	−1.86	139	262	M18070	100606_at
Gbp2, guanylate nucleotide binding protein 2	Immune response (GTPase activity)	3	−1.91	151	298	AJ007970	104597_at
Ccl4, chemokine (C-C motif) ligand 4	Chemotaxis	11	−1.92	163	318	X62502	94146_at
Copeb, core promoter element binding protein	(DNA binding, nucleic acid binding, zinc ion binding)	13	−1.98	190	376	AW049031	98083_at
Cd5, CD5 Ag	(MHC protein binding, exogenous peptide antigen binding, scavenger receptor activity)	19	−2.04	267	541	M15177	93637_at
IL-3, interleukin 3	Immune response (cytokine, growth factor)	11	−2.06	544	1119	K01668	94086_at
cDNA	(Ribosomal_L7Ae; structural constituent of ribosome;1.9e-20)		−2.06	329	680	AV138783	161666_f_at
Rab6ip1, Rab6 interacting protein 1	(Rab interactor activity, protein binding)	7	−2.08	136	286	AJ245569	104108_at
Tgtp, T cell specific GTPase	(GTP binding)	11	−2.10	210	462	L38444	102906_at
IL-7r interleukin 7 receptor	Cell surface receptor-linked signal transduction	15	−2.11	30	63	M29697	99030_at
1110019C08Rik RIKEN cDNA		16	−2.17	121	269	AI838249	160228_at
Ifitm3, interferon-induced transmembrane protein 3	Immune response	7	−2.22	554	1227	AW125390	160253_at
Igtp, interferon γ, induced GTPase	(GTPase activity)	11	−2.22	224	524	U53219	160933_at
2310047C17Rik RIKEN cDNA	Intracellular signaling cascade	19	−2.59	107	276	AA657044	160255_at
IL-13, interleukin 13	Immune response (cytokine, growth factor)	11	−2.75	247	663	M23504	94168_at
Ramp3, receptor (calcitonin) activity modifying protein 3	G protein-coupled receptor protein signaling pathway	11	−2.90	203	588	AJ250491	92368_at
IL-1r2, interleukin 1 receptor, type II	Cell surface receptor linked signal transduction (blocking receptor)	1	−2.93	42	122	X59769	102658_at
Atf3, activating transcription factor 3	Gluconeogenesis, regulation of transcription (DNA-dependent)	1	−2.97	95	281	U19118	104155_f_at
Gbp1, guanylate nucleotide binding protein 1	Immune response (GTPase activity)	3	−3.07	103	295	M55544	95974_at
Lta, lymphotoxin A	Cell growth, maintenance, proliferation; cellular defense	17	−3.11	102	318	M16819	102630_s_at
Icos, inducible T cell co-stimulator	(MHC protein binding, exogenous peptide antigen binding)	1	−3.78	59	220	AB023132	98282_at
IL-17, interleukin 17	(cytokine activity)	1	−3.98	11	54	U35108	99349_at
Gja1, gap junction membrane channel protein alpha 1	Cell communication, cell-cell signaling	10	−4.04	9	36	M63801	100064_f_at
IL-9, interleukin 9	Immune response (cytokine, growth factor)	13	−4.52	51	237	M30136	96574_at
Lmna, lamin A	(protein binding, structural molecule activity)	3	−7.84	84	666	D49733	98059_s_at

[a] Gene name, functional information, and chromosome location (Chr) from National Center for Biotechnology Information EntrezGene (⟨www.ncbi.nlm.nih.gov/Entrez⟩).

[b] Gene expression (arbitrary fluorescence units) are mean values from duplicate experiments for the RXR agonist treatment and vehicle control treatment from matched Affymetrix U74Av2 murine microarray chips; fold change (RXR/control) was calculated for each matched pair, and the mean is reported here. The range of mean signals for these probe sets was from 9 to 3578. The overall mean ± SD was 434 ± 509, and the median (25th, 75th percentile) was 279 (142,518). Of the probe sets showing RXR agonist-induced increases ($n = 68$), the mean and median signal ratios (RXR/vehicle) were 2.62 ± 3.93 and 1.60 (1.40, 1.95) with a range of 1.3 to 24.5. Of those showing decreases ($n = 68$), the mean and median ratios (vehicle/RXR) were 2.00 ∀ 1.01 and 1.60 (1.40, 2.10) with a range of 1.3 to 7.8.

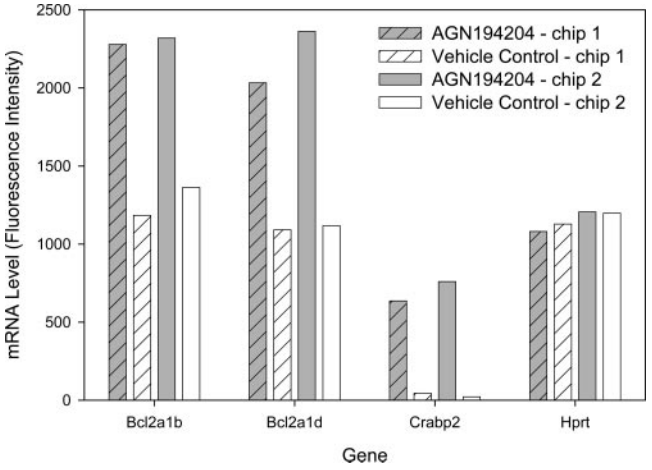

FIGURE 3. mRNA expression from two independent experiments (*chip 1*, *chip 2*) measured by microarray analysis (as described in *Materials and Methods*) following treatment with the RXR agonist AGN194204 (10 nM in 0.1% DMSO) or vehicle control (0.1% DMSO) in naive DO11.10 T lymphocytes sorted to >96% purity 64 h following primary antigenic stimulation with irradiated splenocytes and $OVA_{323–339}$ peptide Ag. The range of mean signals for the 136 probe sets that showed differential expression (see *Results*) was from 9 to 3578. The overall mean ± SD was 434 ± 509, and the median (25th, 75th percentile) was 279 (142, 518). The *Bcl2a1b*, *Bcl2a1d*, and *Crabp2* genes were selected as significantly different in both experiments, whereas the *Hprt* gene was not.

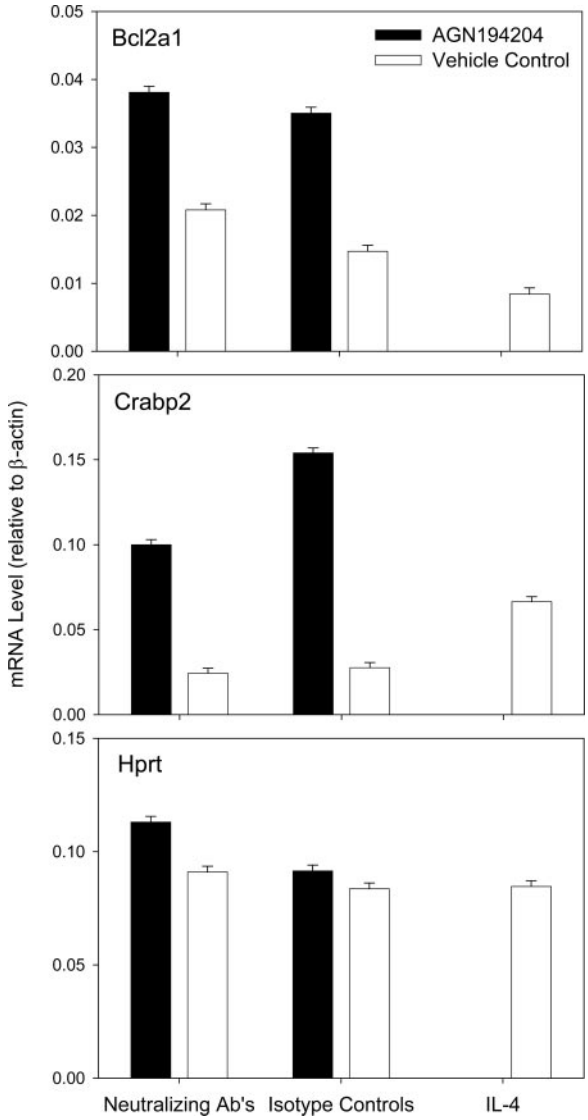

FIGURE 4. mRNA expression of Bcl2a1, Crabp2, and Hprt measured by qRT-PCR in naive DO11.10 T lymphocytes sorted to >96% purity 64 h following primary antigenic stimulation with irradiated splenocytes and $OVA_{323–339}$ peptide Ag. At the time of antigenic stimulation, cultures were treated with the RXR agonist AGN194204 (10 nM in 0.1% DMSO) or vehicle control (0.1% DMSO) either with neutralizing Abs for IL-4, IFN-γ, and IL-12 (neutralizing Abs) or with isotype control Abs (5 μg/ml each) or with IL-4 (10 ng/ml). Results are the mean (±SE) from three independent experiments. Two-way ANOVA comparing RNA levels for the RXR agonist vs vehicle control within Ab treatments showed that RNA levels for the RXR agonist treatment were greater than for the vehicle control for Bcl2a1 and Crabp2 for both Ab treatments ($p < 0.001$). Neither difference was significant ($p > 0.05$) for Hprt RNA.

treatments. Both treatments significantly increased Crabp2 expression over levels seen in the vehicle control (Fig. 5).

Effect of RXR partner ligands on Bcl2a1 RNA expression

RXR may regulate Bcl2a1 expression by forming a heterodimer with a permissive partner receptor. Such receptors include the vitamin D receptor (VDR, NR1I1), thyroid hormone receptor (TR, NR2C), farnesoid X receptor (FXR, NR1H4), liver X receptor (LXR, NR1H), RAR (NR1B), PPAR (NR1C) (47). Thus, treatment of T lymphocytes with the partner ligand alone might increase Bcl2a1 expression. To test this hypothesis, cultures of naive $CD4^+$ DO11.10 T lymphocytes were stimulated with anti-CD3 and anti-CD28 Abs and treated with partner-receptor ligands (100 nM 1,25$(OH)_2$ vitamin D_3, 100 nM 3,3′,5-tri-iodothyronine, 50 μM chenodeoxycholic acid, 5 μM 22(*R*)-hydroxycholesterol, 100 nM all-*trans*-retinoic acid, and 100 nM ciglitazone) in the presence the RXR agonist AGN194204 (100 nM) or vehicle control (0.1% DMSO). In four independent experiments, treatment with AGN194204 alone significantly increased Bcl2a1 expression above the level seen with the vehicle control, as was expected, but none of the partner ligands used alone significantly increased Bcl2a1 expression (data not shown). Treatment with each partner ligand in combination with the RXR agonist also significantly increased Bcl2a1 expression above the level seen with the vehicle control, but none of these combinations significantly increased Bcl2a1 expression above the level seen with AGN194204 alone (data not shown). Thus, these ligands for VDR, TR, FXR, LXR, RAR, and PPAR-γ do not increase Bcl2a1 mRNA under these experimental conditions.

RXR agonist decreases T lymphocyte apoptosis following primary stimulation

Because Bcl2a1 inhibits apoptotic death of naive T lymphocytes following antigenic stimulation, we anticipated that treatment of stimulated T lymphocytes with AGN194204 would also decrease apoptosis. To test this hypothesis we treated $CD4^+$ DO11.10 T lymphocytes (stimulated with anti-CD3 and anti-CD28 Ab) with the RXR agonist AGN194204 or vehicle control (0.1% DMSO), stained cells with PE-labeled annexin V and 7-AAD, and analyzed cells by flow cytometry. The percentage of cells positive for both annexin V and 7-AAD was lower in the RXR agonist-treated cultures at all time points. These differences were statistically significant by two-way ANOVA at 24 h ($p < 0.001$, $n = 3$ experiments) and 48 h ($p < 0.001$, $n = 3$ experiments) but not at 72 h ($n = 2$ experiments) (Fig. 6, *top panel*). The percentage of cells earlier in apoptosis, which stained only positive for annexin V but not for 7-AAD, did not differ at 24 h, but there was significantly less

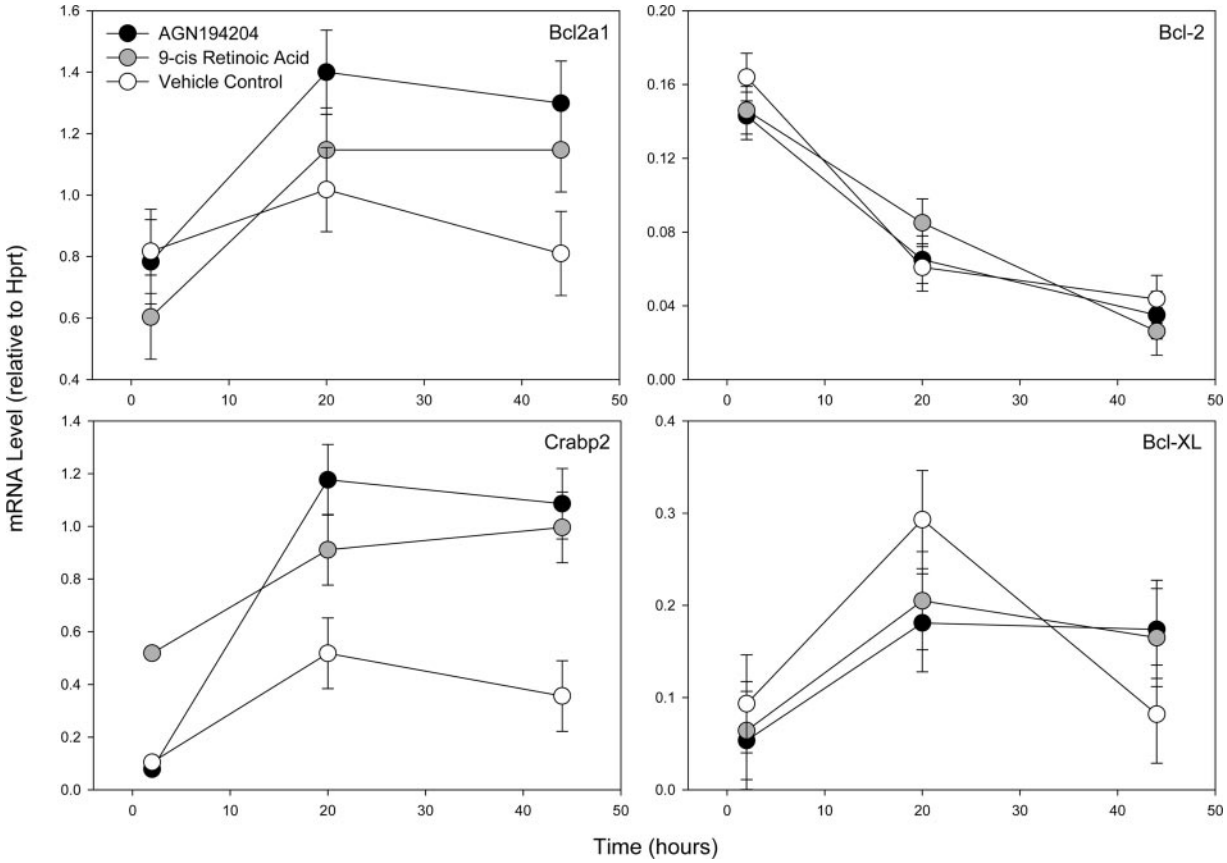

FIGURE 5. Effect of the RXR agonists AGN194204 and 9-*cis*-retinoic acid on *Bcl2a1*, *Bcl2*, *Bcl-x$_L$*, and *Crabp2* gene expression in T lymphocytes. CD4$^+$ T lymphocytes were purified by positive selection from RAG$^{-/-}$ DO11.10 mice. Cells were treated with the RXR agonists AGN194204 (100 nM in 0.1% DMSO), 9-*cis*-retinoic acid (100 nM in 0.1% DMSO), or vehicle (0.1% DMSO) and stimulated with anti-CD3 and anti-CD28 Ab at 0 h. RNA was extracted at 2, 20, and 44 h, and mRNA levels were determined using qRT-PCR. Each data point represents the mean ± SE from five independent experiments. Two-way repeated measures ANOVA of all treatments and time points was used to determine whether the retinoid treatments differed significantly from the vehicle control. Significant differences ($p < 0.05$) for the AGN194204 treatment were seen for Crabp2 ($p = 0.005$) and Bcl2a1 ($p = 0.004$). For 9-*cis*-retinoic acid a significant difference was seen for Crabp2 ($p = 0.009$) and for Bcl2a1 when 24 and 48 h were analyzed separately by a paired *t* test ($p = 0.027$).

apoptosis in cultures treated with the RXR agonist at both 48 and 72 h ($p < 0.001$) (Fig. 6, *bottom panel*). Thus, treatment with the RXR agonist decreased apoptosis of naive T lymphocytes during initial stimulation via the TCR with CD28-mediated costimulation.

Growth factor deficiency may contribute to apoptosis of T lymphocytes following antigenic stimulation. To determine whether growth factor deficiency was a factor in these experiments, we measured the effect of AGN194204 and 9-*cis*-retinoic acid on apoptosis 48 h after stimulation in the presence of IL-2 or IL-4 or without cytokine. The percentage of cells undergoing apoptosis was highest without cytokine (43.6%), significantly lower with IL-2 treatment (41.4%) and (significantly) lower still with IL-4 treatment (37.2%) (Fig. 7). Both AGN194204 and 9-*cis*-retinoic acid significantly decreased the percentage of cells that were positive for both annexin V and 7-AAD with our without cytokine treatment (Fig. 7). Thus, the effect of these RXR agonists on protection from apoptosis is independent of treatment with IL-2 and IL-4.

*Effect of AGN194204 and 9-*cis*-retinoic acid on Bcl2 and Bcl-x$_L$ expression*

Because *Bcl2* and *Bcl-x$_L$* are antiapoptotic genes that act in a manner similar to Bcl2a1, we examined the effect of retinoid treatment on their expression in stimulated T lymphocyte cultures (Fig. 5). Bcl2 expression was highest at 2 h and decreased steadily through 20 and 44 h, but the RXR agonists AGN194204 and 9-*cis*-retinoic acid had no significant effects on Bcl2 expression. Bcl-x$_L$ expression was lowest at 2 h, increased by 20 h, and then decreased slightly by 44 h. Neither RXR agonist significantly affected Bcl-x$_L$ expression (Fig. 5). Thus, these RXR agonists did not affect expression of the antiapoptotic genes *Bcl2* and *Bcl-x$_L$*.

Bcl2a1 RNA expression is higher in nonapoptotic than apoptotic T lymphocytes

Given the direct effect of Bcl2a1 on protection from apoptosis in naive T lymphocytes (17), we anticipated that Bcl2a1 mRNA levels would be higher in nonapoptotic than in apoptotic cells (i.e., the ratio in sorted cells would be >1.0). To confirm this expectation, we stimulated naive DO11.10 T lymphocytes with anti-CD3 and anti-CD28 Abs, separated viable, nonapoptotic cells (annexin V$^-$/7-AAD$^-$) from viable, apoptotic cells (annexin V$^+$/7-AAD$^-$) by cell sorting, and measured Bcl2a1 expression by qRT-PCR 24, 48, and 72 h after stimulation. Cultures were treated with AGN194204 (100 nM) or vehicle control (0.1% DMSO), and one sample from each treatment was sorted and analyzed at each time point. The overall mean ratio (±SD) of Bcl2a1 mRNA levels in nonapoptotic vs apoptotic cells was 3.97 ± 1.20 ($n = 6$), significantly greater than 1.0 ($p = 0.002$ by paired Student's *t* test controlling for time point). The ratios increased over time (2.8, 4.4, and 4.7 at 24, 48, and 72 h) but did not differ by retinoid treatment. Thus, nonapoptotic cells from both treatments had higher Bcl2a1 expression than

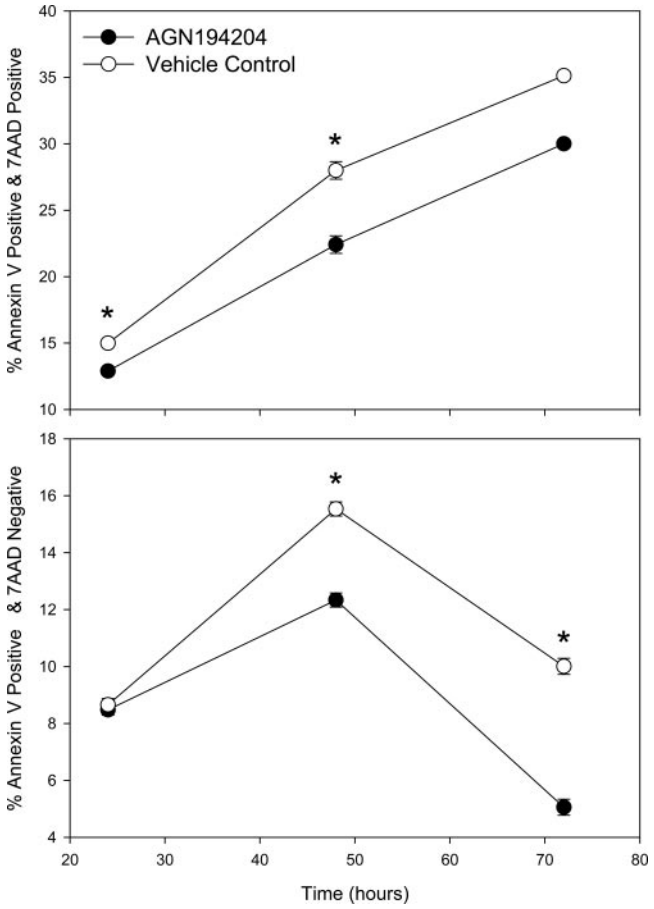

FIGURE 6. Percentage of apoptotic cells among CD4-selected, naive DO11.10 T lymphocytes stimulated with anti-CD3 and anti-CD28 Abs and treated with an RXR agonist (100 nM AGN194204) or vehicle control (0.1% DMSO) (±SE). *Top panel*, Dead cells in the lymphocyte gate (identified by forward scatter, side scatter analysis) were identified by flow cytometric analysis as double positive for annexin V and 7-AAD (annexin V^+-$7AAD^+$). *Bottom panel*, Viable apoptotic cells in the lymphocyte gate were identified as positive for annexin V and negative for 7-AAD (annexin V^+/$7AAD^-$). Data are from three independent experiments. An asterisk (*) indicates significant differences ($p < 0.05$) between treatments at the indicated times as identified by two-way ANOVA.

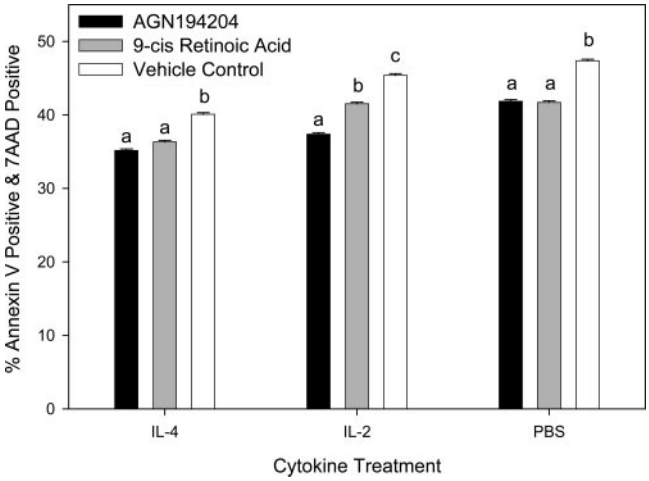

FIGURE 7. Percentage of apoptotic cells among CD4-selected, naive DO11.10 T lymphocytes 48 h after stimulation with anti-CD3 and anti-CD28 Abs (±SE). Apoptotic cells were identified by flow cytometric analysis following annexin V and 7-AAD staining. Cultures were treated with IL-4 (20 ng/ml), IL-2 (50 ng/ml), or vehicle control (PBS), in combination with AGN194204 (100 nM in 0.1% DMSO), 9-*cis*-retinoic acid (100 nM in 0.1% DMSO), or vehicle control (0.1% DMSO). The percentage of apoptotic cells (annexin V^+-$7\text{-}AAD^+$) in the lymphocyte gate by forward scatter and side scatter analysis was measured by flow cytometric analysis. Data are from three independent experiments and were compared by three-way ANOVA (comparing retinoid treatment, cytokine treatment, and experiment). The means for the three cytokine treatments were all significantly different from one another. Within cytokine treatments, bars with different superscript letters (a, b, and c) are significantly different from one another. a, $p \leq 0.001$; b, $p \leq 0.01$; c, $p < 0.05$.

apoptotic cells, consistent with the known antiapoptotic activity of Bcl2a1. Crabp2 mRNA levels were examined and no differences in expression ratios were found between nonapoptotic and apoptotic cells (mean ratio 1.06 ± 0.44, $p = 0.74$), as would be expected for a gene not known to be involved in apoptosis.

*AGN194204 and 9-*cis*-retinoic acid increase transcription from the Bcl2a1 promoter*

Because both AGN194204 and 9-*cis*-retinoic acid increased Bcl2a1 mRNA levels in stimulated T lymphocytes, we wished to determine whether the 5′-regulatory region of Bcl2a1 responds directly to treatment with these RXR agonists. Therefore, we examined the effects of AGN194204 and 9-*cis*-retinoic acid on the ability of 2.0 kb of genomic DNA found immediately upstream of the *Bcl2a1* structural gene to regulate expression of a luciferase reporter gene. This region contains several transcription factor binding sites or importance to regulation of *Bcl2a1* gene expression (39). Because the effect of retinoids on transcription may vary by cell type, we initially attempted to transfect this reporter construct into primary T lymphocytes and a T lymphocyte line (EL4). Efficiencies were very low (data not shown) so we used murine macrophage (Raw) and fibroblast (3T3) cell lines.

In both Raw and 3T3 cells, we found that luciferase activity was increased significantly by treatment with either AGN194204 or 9-*cis*-retinoic acid. As shown in Fig. 8, 9-*cis*-retinoic acid increased luciferase activity by 103% in Raw cells and 91% in 3T3 cells. When an expression plasmid for RXR-α was cotransfected with the reporter plasmid, basal expression in the Raw and 3T3 cells was 70 and 416% higher than without the expression plasmid. Addition of 9-*cis*-retinoic acid again increased luciferase activity in the presence of the reporter plasmid by 136% in Raw cells and by 103% in 3T3 cells. The effect of different concentrations of 9-*cis*-retinoic acid and AGN194204 on transcriptional activity was assessed in 3T3 cells. Both retinoids showed an ability to increase luciferase activity at low concentrations (1 nM for 9-*cis*-retinoic acid and 10 nM for AGN194204) and a dose-response effect was seen at higher concentrations, with the maximum increase being 94% for 9-*cis*-retinoic acid and 52% for AGN194204. Thus, the Bcl2a1 promoter is responsive to retinoids at physiologically relevant concentrations.

Discussion

Many nuclear receptors are expressed in T lymphocytes, including RAR, RXR, PPAR, VDR, ROR-γ (NR1F3), Nur77 (NR4A1), and Nor1 (NR4A3) (7, 10, 48, 49). In TCR-transgenic DO11.10 T lymphocytes, we detected mRNA for RAR-α, RAR-γ, RXR-α, and RXR-β. Protein was also detected for three of these four receptors, with the exception of RAR-γ. It is possible that our Western blot analysis was not sensitive enough to detect RAR-γ, although RNA levels for RAR-α and RAR-γ were similar. Other investigators report the presence of RAR-γ mRNA in T lymphocytes and biological activity for RAR-γ-selective ligands (24). To

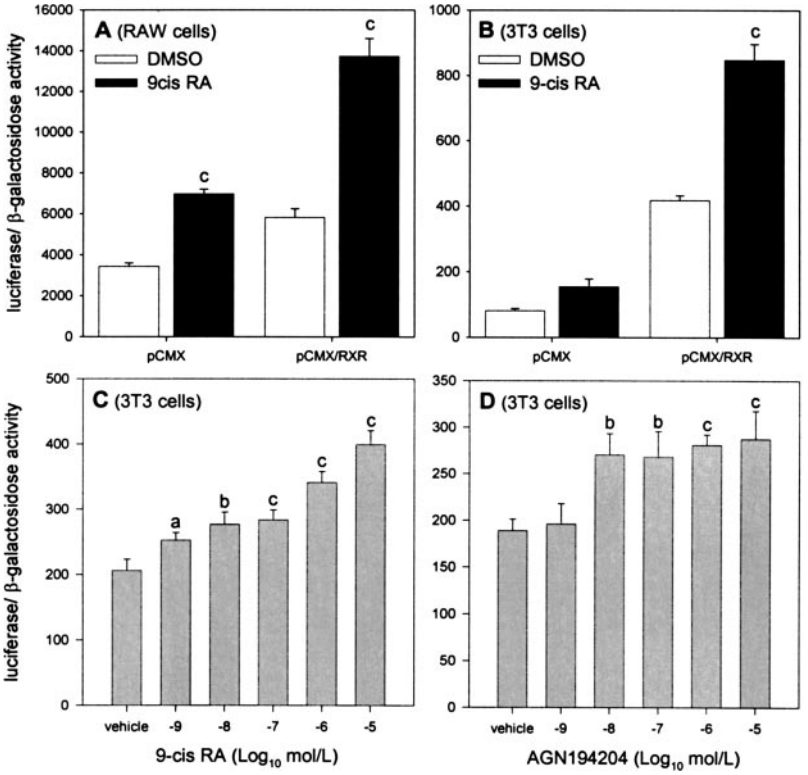

FIGURE 8. Effect of AGN194204 and 9-*cis*-retinoic acid on Bcl2a1 promoter activity. Raw 264.7 cells (*A*) and 3T3 cells (*B–D*) were transfected with the Bcl2a1 promoter linked to the luciferase gene (pGL3/Bcl2a1) and with a constitutive reporter plasmid for β-galactosidase (HSP70-β-galactosidase). Cells were also transfected with an expression vector for rat RXR-α (*pCMX/RXR*) (*C* and *D*; as indicated in *A* and *B*) or with the empty vector (pCMX) (as indicated in *A* and *B*). All transfections were done 1 day before treatment with vehicle (DMSO), 9-*cis*-retinoic acid (9-*cis*-RA), or the RXR agonist AGN194204. Luciferase activity was determined after 24 h and normalized to β-galactosidase activity. Results are representative of at least three independent experiments. Values are expressed as the mean ± SD of triplicate wells. Statistically significant differences (by *t* test or one-way ANOVA) from the vehicle control are indicated by the following superscript letters: a, $p \leq 0.001$; b, $p \leq 0.01$; and c, $p < 0.05$.

our knowledge, RAR-γ protein has not been identified in T lymphocytes by Western blot analysis, thus the significance of RAR-γ RNA levels in these cells remains uncertain.

RAR mRNA levels decreased after antigenic stimulation while RXR mRNA levels increased (with IL-4 treatment) or remained stable, suggesting a requirement for RXR-mediated signaling in developing memory T lymphocytes. Increased RXR-α expression has also been reported following TCR stimulation in proliferating human T lymphocytes (50), although stimulation of resting human T lymphocytes decreases RXR-α mRNA and protein levels (51). The latter situation is more analogous to the present study, although our murine, TCR-transgenic T lymphocytes were activated with APCs and specific Ag, rather than the polyclonal stimulation used in the studies of resting, human peripheral blood T lymphocytes. This difference in stimulation, or the many differences between these murine and human culture systems, may account for the differences in RXR-α expression seen between the two studies.

Our microarray analysis demonstrated that the expression of many genes involved in Th1/Th2 development, cell proliferation, and cell survival were regulated by RXR in naive T lymphocytes during primary antigenic stimulation. Regulation of these genes may occur directly or indirectly. Many genes directly regulated by RXR agonists, including *Crabp2* and *Scd1*, were identified in these experiments. Although many microarray studies use cell lines, which may not adequately represent the same cell type in vivo, or use tissues from treated animals, which contain many cell types, our study used primary cells that were purified to >96% homogeneity before RNA extraction. Thus, we have a high degree of confidence that the genes identified by this method are expressed in primary T lymphocytes and that differences in mRNA levels reflect actual differences in gene expression in these cells, rather than differences in frequencies of cells in a mixed population.

One of the genes induced by AGN194204 in our microarray studies was *Bcl2a1*. This gene is induced by TCR stimulation (33) and treatment with AGN194204 enhanced Bcl2a1 expression above levels seen by TCR stimulation alone, using either APCs and peptide Ag or ant-CD3 plus anti-CD28 Abs. RAR and RXR agonists can also affect expression of IL-12 by APCs and of IL-4 and IFN-γ by T lymphocytes (2). However, neutralizing Abs were used to rule out an effect of these cytokines on Bcl2a1 expression in our experiments. Because AGN194204 is a synthetic agonist, we also tested the naturally occurring RXR agonist 9-*cis*-retinoic acid for the ability to increase Bcl2a1 RNA expression. 9-*cis*-retinoic acid also increased Bcl2a1 mRNA levels, although the magnitude of the response was not as great as seen with equal concentrations of AGN194204. This may be because the binding affinity and transactivation activity (EC_{50} concentration) are 10-fold lower for AGN194204 than for 9-*cis*-retinoic acid (38), making AGN194204 a more potent agonist. These results confirm that Bcl2a1 mRNA levels are greater in primary T lymphocyte cultures treated with RXR agonists, and are consistent with an RXR-mediated increase in transcription.

To determine whether AGN194204 and 9-*cis*-retinoic acid directly regulate transcription from the Bcl2a1 promoter, we conducted experiments using a luciferase reporter construct containing 2.0 kb of cloned genomic DNA from the 5′ noncoding region of the *Bcl2a1a* gene (39). These experiments confirmed that both AGN194204 and 9-*cis*-retinoic acid increased luciferase activity in 3T3 and Raw cells. To our knowledge, these are the first data to directly indicate that retinoids regulate *Bcl2a1* gene expression. Additional experiments will be needed to determine how these agonists affect transcription. It is possible that an RXR homodimer or heterodimer binds to an appropriate response element in the Bcl2a1 promoter and thereby enhance transcription (52). Preliminary examination of the Bcl2a1 promoter revealed a nearly canonical DR4 element (data not shown), but agonists for two nuclear receptors that typically act via a DR4 element, TR and LXR, did not significantly enhance Bcl2a1 expression in the present study, nor did the ligands for PPAR-γ, FXR, RAR, or VDR enhance Bcl2a1 expression. These negative results suggest that these

partner receptors are not involved in regulation of Bcl2a1 expression. However, the results are not definitive because these experiments were conducted using cell culture medium containing FBS, which may contain partner ligands (e.g., all-*trans*-retinoic acid or thyroid hormone) that could obscure enhancement of Bcl2a1 expression by added partner ligands.

Rather than bind to a response element, RXR could interact directly with transcription factors, such as NF-κB, and thereby affect transcription (53). This is plausible because Bcl2a1 transcription is regulated by NF-κB (35), but such interactions often result in decreased rather than increased transcription as a result of competition among different transcription factors for access to the transcription complex. However, a recent study revealed that retinoids induced apoptosis via an RAR-α/RXR pathway in immature dendritic cells. But when dendritic cells were also stimulated with inflammatory signals cell survival, maturation, and Ag presentation were enhanced. The latter effects depended on RXR were independent of RAR and appeared to be mediated by increased nuclear translocation of NF-κB (54). Because Bcl2a1 transcription is increased by NF-κB binding to its promoter following TCR engagement (39), the same RXR-mediated enhancement of NF-κB activity could be responsible for increased Bcl2a1 expression in the present experiments. Further work is needed to address this hypothesis.

Previous studies have found that all-*trans*-retinoic acid increases Bcl2a1 mRNA levels and decreases apoptosis in the NB4 and PBL985 acute promyelocytic leukemia cell lines (55–58). In a more recent study with PBL985 cells, 9-*cis*-retinoic acid increased Bcl2a1 expression but the RXR-selective agonist SR11237 did not (59). These results are consistent with our findings that 9-*cis*-retinoic acid increased Bcl2a1 mRNA levels in both the leukemia cell lines and in primary T lymphocytes. However, in the present study, all-*trans*-retinoic acid did not increase Bcl2a1 mRNA without coadministration of AGN194204. It is possible that RAR was not expressed at a sufficient level to mediate these effects in our experiments as expression decreased after antigenic stimulation. In addition, the profile of coactivator and corepressor proteins undoubtedly differs between promyelocytic leukemia cells and primary $CD4^+$ T lymphocytes. Such differences could account for the disparate results with RAR and RXR agonists between these two sets of experiments.

Different mechanisms regulate T lymphocyte survival and loss during the different phases of the immune response. During the postactivation phase, stimulated T lymphocytes are susceptible to activation-induced cell death by restimulation via the TCR. Apoptosis can then be induced by binding of Fas ligand to Fas expressed on the surface of susceptible cells. T cell hybridomas are used as models of activation-induced cell death (18). In this system, retinoic acid inhibits Fas-mediated apoptosis by decreasing cell surface Fas ligand expression (19–22). However, this mechanism is of limited importance following primary antigenic stimulation as naive T lymphocytes are resistant to activation-induced cell death (18, 46).

Survival of naive T lymphocytes during the first few days following primary stimulation is enhanced by several factors, including the activity of growth-promoting cytokines such as IL-2 and IL-4 (15, 18). Although cytokine withdrawal can induce apoptosis and retinoids are known to affect IL-2 production and receptor expression (60, 61), addition of IL-2 and IL-4 to cultures in the present studies did not alter the antiapoptotic effects of AGN194204 and 9-*cis*-retinoic acid. This result was not unexpected because these cytokines do not induce Bcl2a1 expression in naive T lymphocytes, although IL-4 can induce Bcl2 expression (17).

Stimulation of naive T lymphocytes via the TCR, with appropriate costimulation, induces expression of prosurvival Bcl2 family members Bcl2a1 and Bcl-x_L (17, 46, 62). Bcl2a1 mRNA expression peaks in the first 24 h following stimulation than decreases to basal levels within 3 days, while Bcl2a1 protein is detectable on days 1 through 3, with a peak on day 2 (17, 46). Transgenic expression of Bcl2a1a using the Lck promoter (which is stimulated by activation of the TCR) enhances survival of stimulated T lymphocytes for at least 3 days (46). These findings are similar to our observations that RXR agonists prolong Bcl2a1 expression 2 and 3 days after stimulation, with corresponding decreases in apoptosis. Our observation that expression of RNA for Bcl2 and Bcl-x_L was not increased by retinoid treatment supports our hypothesis that RXR agonists diminish apoptosis by increasing Bcl2a1 expression. Additional experiments to block Bcl2a1 expression or function following RXR stimulation will be needed to confirm this hypothesis.

In conclusion, we have demonstrated that RXR agonists increase Bcl2a1 expression, decrease apoptosis, and increase cell number following primary antigenic stimulation of naive $CD4^+$ T lymphocytes. In addition, these agonists induce the transcriptional activity of Bcl2a1 using a luciferase reporter gene. Thus, RXR appears to promote the survival of T lymphocytes during initial exposure to Ag. Retinoids may be pro- or antiapoptotic, depending on the type of cell involved, with proapoptotic effects often being seen in transformed cells (63), including T cell lymphomas (27). In the present experiments, RXR ligands play an antiapoptotic role, as has been seen recently for RXR and LXR in macrophages during antibacterial responses (64). Thus, ligands for RXR appear to promote cell survival during the initiation and effector phases of both innate and adaptive immune responses. This role should be of benefit in fighting infectious diseases but may be a mixed blessing during chronic inflammatory or autoimmune disease when increased cell survival may mean more severe disease. Further work is needed on the specific mechanisms of action of RXR and its partner receptors to understand how the immune response may be modified by dietary and pharmacologic ligands for these receptors.

Acknowledgments

We thank Xiaowen Jiang and Alina Wettstein for technical assistance, Ling Zhao for sharing specific primer sequences for BCL-x_L, and Martin Privalsky for commenting on the manuscript. We also thank Daniel Hwang for helpful discussions and comments on these experiments.

Disclosures

The authors have no financial conflict of interest.

References

1. Green, H. N., and Mellanby, E. Vitamin A as an anti-infective agent. 1928. *Br. Med. J.* 2: 691–696.
2. Stephensen, C. B. 2001. Vitamin A, infection, and immune function. *Annu. Rev. Nutr.* 21: 167–192.
3. Duester, G. 2000. Families of retinoid dehydrogenases regulating vitamin A function: production of visual pigment and retinoic acid. *Eur. J. Biochem.* 267: 4315–4324.
4. Bastien, J., and C. Rochette-Egly. 2004. Nuclear retinoid receptors and the transcription of retinoid-target genes. *Gene* 328: 1–16.
5. Levin, A. A., L. J. Sturzenbecker, S. Kazmer, T. Bosakowski, C. Huselton, G. Allenby, J. Speck, C. Kratzeisen, M. Rosenberger, A. Lovey, et al. 1992. 9-*Cis*-retinoic acid stereoisomer binds and activates the nuclear receptor RXRα. *Nature* 355: 359–361.
6. Heyman, R. A., D. J. Mangelsdorf, J. A. Dyck, R. B. Stein, G. Eichele, R. M. Evans, and C. Thaller. 1992. 9-*Cis*-retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* 68: 397–406.
7. Winoto, A., and D. R. Littman. 2002. Nuclear hormone receptors in T lymphocytes. *Cell* 109(Suppl.): S57–S66.
8. Daynes, R. A., and D. C. Jones. 2002. Emerging roles of PPARs in inflammation and immunity. *Nat. Rev. Immunol.* 2: 748–759.
9. Jones, D. C., B. M. Manning, and R. A. Daynes. 2002. A role for the peroxisome proliferator-activated receptor α in T cell physiology and ageing immunobiology. *Proc. Nutr. Soc.* 61: 363–369.
10. Szondy, Z., U. Reichert, and L. Fesus. 1998. Retinoic acids regulate apoptosis of T lymphocytes through an interplay between RAR and RXR receptors. *Cell Death Differ.* 5: 4–10.
11. Cantorna, M. T., Y. Zhu, M. Froicu, and A. Wittke. 2004. Vitamin D status, 1,25-dihydroxyvitamin D_3, and the immune system. *Am. J. Clin. Nutr.* 80: 1717S–1720S.

12. Stephensen, C. B., R. Rasooly, X. Jiang, M. A. Ceddia, C. T. Weaver, R. A. Chandraratna, and R. P. Bucy. 2002. Vitamin A enhances in vitro Th2 development via retinoid X receptor pathway. *J. Immunol.* 168: 4495–4503.
13. Mandal, M., C. Borowski, T. Palomero, A. A. Ferrando, P. Oberdoerffer, F. Meng, A. Ruiz-Vela, M. Ciofani, J. C. Zuniga-Pflucker, I. Screpanti, et al. 2005. The *BCL2A1* gene as a pre-T cell receptor-induced regulator of thymocyte survival. *J. Exp. Med.* 201: 603–614.
14. Kim, H., Y. N. Kim, H. Kim, and C. W. Kim. 2005. Oxidative stress attenuates Fas-mediated apoptosis in Jurkat T cell line through Bfl-1 induction. *Oncogene* 24: 1252–1261.
15. Opferman, J. T., and S. J. Korsmeyer. 2003. Apoptosis in the development and maintenance of the immune system. *Nat. Immunol.* 4: 410–415.
16. Williams, O., and H. J. Brady. 2001. The role of molecules that mediate apoptosis in T cell selection. *Trends Immunol.* 22: 107–111.
17. Verschelde, C., T. Walzer, P. Galia, M. C. Biemont, L. Quemeneur, J. P. Revillard, J. Marvel, and N. Bonnefoy-Berard. 2003. A1/Bfl-1 expression is restricted to TCR engagement in T lymphocytes. *Cell Death Differ.* 10: 1059–1067.
18. Lenardo, M., K. M. Chan, F. Hornung, H. McFarland, R. Siegel, J. Wang, and L. Zheng. 1999. Mature T lymphocyte apoptosis: immune regulation in a dynamic and unpredictable antigenic environment. *Annu. Rev. Immunol.* 17: 221–253.
19. Yang, Y., M. Mercep, C. F. Ware, and J. D. Ashwell. 1995. Fas and activation-induced Fas ligand mediate apoptosis of T cell hybridomas: inhibition of Fas ligand expression by retinoic acid and glucocorticoids. *J. Exp. Med.* 181: 1673–1682.
20. Yang, Y., S. Minucci, K. Ozato, R. A. Heyman, and J. D. Ashwell. 1995. Efficient inhibition of activation-induced Fas ligand up-regulation and T cell apoptosis by retinoids requires occupancy of both retinoid X receptors and retinoic acid receptors. *J. Biol. Chem.* 270: 18672–18677.
21. Bissonnette, R. P., T. Brunner, S. B. Lazarchik, N. J. Yoo, M. F. Boehm, D. R. Green, and R. A. Heyman. 1995. 9-*Cis*-retinoic acid inhibition of activation-induced apoptosis is mediated via regulation of fas ligand and requires retinoic acid receptor and retinoid X receptor activation. *Mol. Cell. Biol.* 15: 5576–5585.
22. Cui, H., D. H. Sherr, M. el-Khatib, K. Matsui, D. J. Panka, A. Marshak-Rothstein, and S. T. Ju. 1996. Regulation of T cell death genes: selective inhibition of FasL- but not Fas-mediated function. *Cell. Immunol.* 167: 276–284.
23. Toth, R., E. Szegezdi, U. Reichert, J. M. Bernardon, S. Michel, P. Ancian, K. Kis-Toth, Z. Macsari, L. Fesus, and Z. Szondy. 2001. Activation-induced apoptosis and cell surface expression of Fas^{CD95} ligand are reciprocally regulated by retinoic acid receptor α and γ and involve nur77 in T cells. *Eur. J. Immunol.* 31: 1382–1391.
24. Toth, B., K. Ludanyi, I. Kiss, U. Reichert, S. Michel, L. Fesus, and Z. Szondy. 2004. Retinoids induce Fas^{CD95} ligand cell surface expression via RARγ and nur77 in T cells. *Eur. J. Immunol.* 34: 827–836.
25. Szondy, Z., U. Reichert, J. M. Bernardon, S. Michel, R. Toth, P. Ancian, E. Ajzner, and L. Fesus. 1997. Induction of apoptosis by retinoids and retinoic acid receptor γ-selective compounds in mouse thymocytes through a novel apoptosis pathway. *Mol. Pharmacol.* 51: 972–982.
26. Iwata, M., M. Mukai, Y. Nakai, and R. Iseki. 1992. Retinoic acids inhibit activation-induced apoptosis in T cell hybridomas and thymocytes. *J. Immunol.* 149: 3302–3308.
27. Wang, K. C., A. L. Cheng, S. E. Chuang, H. C. Hsu, and I. J. Su. 2000. Retinoic acid-induced apoptotic pathway in T cell lymphoma: identification of four groups of genes with differential biological functions. *Exp. Hematol.* 28: 1441–1450.
28. Murphy, K. M., A. B. Heimberger, and D. Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of $CD4^+CD8^+TCR^{low}$ thymocytes in vivo. *Science* 250: 1720–173.
29. Lin, E. Y., A. Orlofsky, M. S. Berger, and M. B. Prystowsky. 1993. Characterization of A1, a novel hemopoietic-specific early-response gene with sequence similarity to bcl-2. *J. Immunol.* 151: 1979–1988.
30. Lin, E. Y., A. Orlofsky, H. G. Wang, J. C. Reed, and M. B. Prystowsky. 1996. A1, a Bcl-2 family member, prolongs cell survival and permits myeloid differentiation. *Blood* 87: 983–992.
31. Orlofsky, A., R. D. Somogyi, L. M. Weiss, and M. B. Prystowsky. 1999. The murine antiapoptotic protein A1 is induced in inflammatory macrophages and constitutively expressed in neutrophils. *J. Immunol.* 163: 412–419.
32. Hamasaki, A., F. Sendo, K. Nakayama, N. Ishida, I. Negishi, K. Nakayama, and S. Hatakeyama. 1998. Accelerated neutrophil apoptosis in mice lacking A1-a, a subtype of the bcl-2-related A1 gene. *J. Exp. Med.* 188: 1985–1992.
33. Tomayko, M. M., J. A. Punt, J. M. Bolcavage, S. L. Levy, D. M. Allman, and M. P. Cancro. 1999. Expression of the Bcl-2 family member A1 is developmentally regulated in T cells. *Int. Immunol.* 11: 1753–1761.
34. Lee, H. H., H. Dadgostar, Q. Cheng, J. Shu, and G. Cheng. 1999. NF-κB-mediated up-regulation of Bcl-x and Bfl-1/A1 is required for CD40 survival signaling in B lymphocytes. *Proc. Natl. Acad. Sci. USA* 96: 9136–9141.
35. Edelstein, L. C., L. Lagos, M. Simmons, H. Tirumalai, and C. Gelinas. 2003. NF-κB-dependent assembly of an enhanceosome-like complex on the promoter region of apoptosis inhibitor Bfl-1/A1. *Mol. Cell. Biol.* 23: 2749–2761.
36. Marrack, P., R. Shimonkevitz, C. Hannum, K. Haskins, and J. Kappler. 1983. The major histocompatibility complex-restricted antigen receptor on T cells. IV. An antiidiotypic antibody predicts both antigen and I-specificity. *J. Exp. Med.* 158: 1635–1646.
37. Iqbal, N., J. R. Oliver, F. H. Wagner, A. S. Lazenby, C. O. Elson, and C. T. Weaver. 2002. T helper 1 and T helper 2 cells are pathogenic in an antigen-specific model of colitis. *J. Exp. Med.* 195: 71–84.
38. Vuligonda, V., S. M. Thacher, and R. A. Chandraratna. 2001. Enantioselective syntheses of potent retinoid X receptor ligands: differential biological activities of individual antipodes. *J. Med. Chem.* 44: 2298–2303.
39. Grumont, R. J., I. J. Rourke, and S. Gerondakis. 1999. Rel-dependent induction of A1 transcription is required to protect B cells from antigen receptor ligation-induced apoptosis. *Genes Dev.* 13: 400–411.
40. Iwata, M., Y. Eshima, and H. Kagechika. 2003. Retinoic acids exert direct effects on T cells to suppress Th1 development and enhance Th2 development via retinoic acid receptors. *Int. Immunol.* 15: 1017–1025.
41. Hoag, K. A., F. E. Nashold, J. Goverman, and C. E. Hayes. 2002. Retinoic acid enhances the T helper 2 cell development that is essential for robust antibody responses through its action on antigen-presenting cells. *J. Nutr.* 132: 3736–3739.
42. Samuel, W., R. K. Kutty, S. Nagineni, J. S. Gordon, S. M. Prouty, R. A. Chandraratna, and B. Wiggert. 2001. Regulation of stearoyl coenzyme A desaturase expression in human retinal pigment epithelial cells by retinoic acid. *J. Biol. Chem.* 276: 28744–28750.
43. Durand, B., M. Saunders, P. Leroy, M. Leid, and P. Chambon. 1992. All-*trans*- and 9-*cis*-retinoic acid induction of CRABPII transcription is mediated by RAR-RXR heterodimers bound to DR1 and DR2 repeated motifs. *Cell* 71: 73–85.
44. Szondy, Z., U. Reichert, J. M. Bernardon, S. Michel, R. Toth, E. Karaszi, and L. Fesus. 1998. Inhibition of activation-induced apoptosis of thymocytes by all-*trans*- and 9-*cis*-retinoic acid is mediated via retinoic acid receptor α. *Biochem. J.* 331(Pt. 3): 767–774.
45. Hatakeyama, S., A. Hamasaki, I. Negishi, D. Y. Loh, F. Sendo, K. Nakayama, and K. Nakayama. 1998. Multiple gene duplication and expression of mouse bcl-2-related genes, A1. *Int. Immunol.* 10: 631–637.
46. Gonzalez, J., A. Orlofsky, and M. B. Prystowsky. 2003. A1 is a growth-permissive antiapoptotic factor mediating postactivation survival in T cells. *Blood* 101: 2679–2685.
47. Chawla, A., J. J. Repa, R. M. Evans, and D. J. Mangelsdorf. 2001. Nuclear receptors and lipid physiology: opening the X-files. *Science* 294: 1866–1870.
48. Jones, D. C., X. Ding, and R. A. Daynes. 2002. Nuclear receptor peroxisome proliferator-activated receptor α (PPARα) is expressed in resting murine lymphocytes: the PPARα in T and B lymphocytes is both transactivation and transrepression competent. *J. Biol. Chem.* 277: 6838–6845.
49. O'Kelly, J., J. Hisatake, Y. Hisatake, J. Bishop, A. Norman, and H. P. Koeffler. 2002. Normal myelopoiesis but abnormal T lymphocyte responses in vitamin D receptor knockout mice. *J. Clin. Invest.* 109: 1091–1099.
50. Ishaq, M., M. Fan, and V. Natarajan. 2000. Accumulation of RXRα during activation of cycling human T lymphocytes: modulation of RXRE transactivation function by mitogen-activated protein kinase pathways. *J. Immunol.* 165: 4217–4225.
51. Ishaq, M., Y. M. Zhang, and V. Natarajan. 1998. Activation-induced down-regulation of retinoid receptor RXRα expression in human T lymphocytes: role of cell cycle regulation. *J. Biol. Chem.* 273: 21210–21216.
52. Szanto, A., V. Narkar, Q. Shen, I. P. Uray, P. J. Davies, and L. Nagy. 2004. Retinoid X receptors: X-ploring their (patho)physiological functions. *Cell Death Differ.* 11(Suppl. 2): S126–S143.
53. Na, S. Y., B. Y. Kang, S. W. Chung, S. J. Han, X. Ma, G. Trinchieri, S. Y. Im, J. W. Lee, and T. S. Kim. 1999. Retinoids inhibit interleukin-12 production in macrophages through physical associations of retinoid X receptor and NFκB. *J. Biol. Chem.* 274: 7674–7680.
54. Geissmann, F., P. Revy, N. Brousse, Y. Lepelletier, C. Folli, A. Durandy, P. Chambon, and M. Dy. 2003. Retinoids regulate survival and antigen presentation by immature dendritic cells. *J. Exp. Med.* 198: 623–634.
55. Jing, Y., L. Xia, and S. Waxman. 2002. Targeted removal of PML-RARα protein is required prior to inhibition of histone deacetylase for overcoming all-*trans*-retinoic acid differentiation resistance in acute promyelocytic leukemia. *Blood.* 100: 1008–1013.
56. Jing, Y., L. Wang, L. Xia, G. Q. Chen, Z. Chen, W. H. Miller, and S. Waxman. 2001. Combined effect of all-*trans*-retinoic acid and arsenic trioxide in acute promyelocytic leukemia cells in vitro and in vivo. *Blood* 97: 264–269.
57. Liu, T. X., J. W. Zhang, J. Tao, R. B. Zhang, Q. H. Zhang, C. J. Zhao, J. H. Tong, M. Lanotte, S. Waxman, S. J. Chen, et al. 2000. Gene expression networks underlying retinoic acid-induced differentiation of acute promyelocytic leukemia cells. *Blood* 96: 1496–1504.
58. Altucci, L., A. Rossin, W. Raffelsberger, A. Reitmair, C. Chomienne, and H. Gronemeyer. 2001. Retinoic acid-induced apoptosis in leukemia cells is mediated by paracrine action of tumor-selective death ligand TRAIL. *Nat. Med.* 7: 680–686.
59. Yin, W., W. Raffelsberger, and H. Gronemeyer. 2005. Retinoic acid determines life span of leukemic cells by inducing antagonistic apoptosis-regulatory programs. *Int J. Biochem. Cell Biol.* 37: 1696–1708.
60. Gorgun, G., and F. Foss. 2002. Immunomodulatory effects of RXR rexinoids: modulation of high-affinity IL-2R expression enhances susceptibility to denileukin diftitox. *Blood* 100: 1399–1403.
61. Ertesvag, A., N. Engedal, S. Naderi, and H. K. Blomhoff. 2002. Retinoic acid stimulates the cell cycle machinery in normal T cells: involvement of retinoic acid receptor-mediated IL-2 secretion. *J. Immunol.* 169: 5555–5563.
62. Lee, H. W., S. J. Park, B. K. Choi, H. H. Kim, K. O. Nam, and B. S. Kwon. 2002. 4-1BB promotes the survival of $CD8^+$ T lymphocytes by increasing expression of Bcl-x_L and Bfl-1. *J. Immunol.* 169: 4882–4888.
63. Altucci, L., E. Wilhelm, and H. Gronemeyer. 2004. Leukemia: beneficial actions of retinoids and rexinoids. *Int. J. Biochem. Cell Biol.* 36: 178–182.
64. Valledor, A. F., L. C. Hsu, S. Ogawa, D. Sawka-Verhelle, M. Karin, and C. K. Glass. 2004. Activation of liver X receptors and retinoid X receptors prevents bacterial-induced macrophage apoptosis. *Proc. Natl. Acad. Sci. USA* 101: 17813–17818.